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BACTERIAL AND MYCOTIC INFECTIONS OF MAN

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Preface to the Third Edition

The present volume does not differ in organization or in point of view from the first and the second editions of *Bacterial and Mycotic Infections of Man* However, while the structure of the book has remained unchanged the text has been almost completely rewritten Because of the amount of new knowledge to be described and the large number of new contributors it has been difficult not to increase the size of the book This has been achieved by reducing somewhat the length of bibliographies for each chapter In certain cases the reader may find it helpful to consult the more extensive list of references printed in the first and the second editions

A word should be said concerning Chapter 2 (The Evolution and the Ecology of Microbial Diseases) In this new chapter I have tried to express the view that the ability of microorganisms to produce pathologic changes is under the influence of large biologic forces as yet poorly understood which do not necessarily manifest themselves in the form of recognized immunochemical reactions Some of the contributors to the book do not entirely share my point of view in this matter, and it is certain indeed that many statements in Chapter 2 cannot be supported by convincing evidence The responsibility for these statements is entirely mine and I wish to thank my colleagues for allowing me to preface their factual presentations with speculative concepts

RENE J DUBOS

The Rockefeller Institute
New York

Preface to the First Edition

This volume was designed to convey to the medical student—and we hope also to the practitioner of medicine—some knowledge of the bacteria, actinomycetes and molds pathogenic for man as well as of the phenomena which characterize the infectious process Infections caused by viruses and rickettsiae are treated in a companion volume edited by Dr T M Rivers

Medical microbiology is the study of host parasite relationships and not that of micro organisms alone considered as independent living agents It is concerned with those aspects of the structure and the properties of parasites which play a part in their pathogenic behavior and with the multiple manifestations of the response of the invaded host to their constituents and products The general chapters of this treatise are therefore devoted to the facts and the problems concerning parasite and host which have a bearing—often immediate but at times only potential and remote—on infectious disease

A few words may be necessary to justify the order in which the different pathogenic agents are described in subsequent chapters This order was adopted to illustrate by the extensive treatment of a few selected examples the multiple facets of the problem of infection Thus the diphtheria bacillus is discussed first to introduce the concept of toxemia and of antitoxic immunity As a counterpart, pneumococcus infections are then selected to emphasize the problems of antibacterial immunity Streptococci on the other hand lend themselves to the demonstration that a given microbial agent can exhibit multiple pathogenic potentialities and that tissues can respond in many different ways to its presence Tuberculosis illustrates particularly well the acute (exudative) and chronic (proliferative) pathologic processes accompanying infection, and the altered reactivity of the body (allergy) which results from previous exposure to the bacillus All these aspects of the infectious process appear in more or less modified form in the other microbial diseases and give to each of them its peculiar character

This treatise is the result of the co-operative effort of many experts and naturally reflects their individual outlooks I wish to thank them all in particular for their willingness to aim at some measure of uniformity in our common undertaking The National Foundation for Infantile Paralysis has given generous financial support to the preparation of the book and shares with us the hope that it may contribute something to the understanding of the general problems of infection

RENE J DUBOS

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Contents

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1

A Synopsis of the History of Medical Bacteriology*

The history of bacteriology is brief but crowded with infinitely varied significance The discoveries and the applications of less than 100 years did more than modify the conceptions and the theories built by the scientific endeavors of preceding centuries they formed a freshly new branch of biologic science *bacteriology* and such great progress was made that there emerged from it the two further highly specialized disciplines of immunology and virology

The stimulus to this phenomenal advance was the establishment of the bacterial cause of infectious disease and with this elucidation came the introduction of exact diagnosis by etiology of specific therapy and of preventive medicine all founded on verifiable fact The profound reformation of medical thought required by the new knowledge of bacteriology was brought about only by a bitter struggle against almost unbelievable opposition but out of it arose the beginnings of experimental medicine Thus bacteriology did not become merely a useful helping hand but the guiding finger and wrought such changes in human health alone that if it be a benefit it must at least equal the contribution to human welfare of any other branch of science To achieve this it has changed and continues still to change the order of importance of various diseases as the cause of death in different age periods Diseases such as typhoid fever diphtheria and pneumonia as examples have been reduced from prevalence with a high mortality to almost a rarity So too the scourge of epidemics has been changed to a threat of danger manifest only if the required precautions are neglected and the fatal menace of some diseases has been softened Meanwhile in the course of the lives of people living today the marvelous achievement of modern surgery was made possible by lifting it out of the despond of laudable pus into the security of asepsis These are only general instances to give point to greater hope for the future

Through the ages humanity of all races used the products of fermentation in various forms of food and drink or for the making of desirable utilities without an inkling of the processes involved The relation of the causes of fermentation and of infectious disease was hardly even suspected for some 2 centuries after bacteria were first definitely seen and figured by Leeuwenhoek (1676) and though he did describe them in pus his discoveries stirred interest in heterogenesis rather than disease Of course it was recognized that certain diseases were catching and that some conferred immunity from a second attack but the idea of contagion passing from one indi

* The names and the dates given relate to the initiation of major trends or developments of medical bacteriology

vidual to another made evident by common observation in plague and syphilis was completely missed in many another disease and was almost certainly exaggerated in leprosy Fracastoro in his book on contagion (1546) was probably the first to indicate that infection itself is composed of minute and insensible particles and proceeds from them' and he wondered whether all contagion may not be a putrefaction he recognized that the infection is the same for him who has received or has given the infection also we speak of infection when the same virus has *touched one or the other*

Much can be read into the early speculative writings, and it is well to be cautious in interpreting them for in most instances it seems evident that the words and the phrases used should not be accepted strictly in their modern meaning Kircher (1658) probably the first to make direct microscopic studies of disease examined putrefying materials and even blood from plague patients to postulate animated corpuscles which constitute the effluvia and scatter new seeds of contagion He supposed a tenacity of life in them and that it is difficult to wash them away, so he recommended burning in the fire clothing and household goods infected with the contagion The immutable specificity of contagious diseases was indicated by the practice of variolation, but it was forcefully expressed by Thomas Fuller (1654 1734) who said that one could not change into another any more than a Hen can breed a Duck and he emphasized it further by saying consequently one Sort cannot be a Preservative against any other Sort'

Speculation on what might have happened is futile but Spallanzani (1775) seems to have only barely failed from revealing the science of bacteriology in the course of his efforts to disprove heterogenesis as upheld by Needham Spallanzani recognized and grew bacteria in sterilized media he discovered forms which grew when deprived of air and he discovered 'germs which we now call endospores of a greater resistance to heat than the forms they gave rise to By his rigid maintenance of conditions of experiment for his intention he failed to inoculate his media selectively on purpose and to realize the full general significance of his discoveries It had all to be re*discovered nearly a century later by Pasteur*, who also took pains during his studies on fermentation to disprove heterogenesis as maintained by Bastian and by Pouchet without losing sight of the singularly far reaching importance of his discoveries

Meanwhile the empirical method of probing the cause of infectious disease proceeded, with a diversity of observations and experiment too extensive to enumerate Outstanding among these, because of their subsequent influence, are John Hunter's ill fated self inoculation with syphilis (1767) from a case *of gonorrhea and Jenner s introduction of vac*cination (1796) against smallpox, using material from naturally acquired cowpox Hunter s experiment was a grave misfortune to himself, and, because he did not recognize a mixed infection, the belief persisted for very many years that syphilis and gonorrhea were the same disease a warning we cannot ignore today Jenner s triumphant success in substantiating a popular belief resulted in widespread vaccination which reduced an almost world wide disease to insignificance The fatal propensity of smallpox is not better illustrated than by its ravages among the Indians of Canada and the United States in 1780 and 1869 and 1870 when whole tribes perished, and others were decimated these epidemics also provide strong evidence of the efficacy of vaccination when properly done and of its failure when care is insufficient Of the same *order of general importance were the insist*ence of Holmes (1843) and Semmelweis (1847 1849) and before them Charles White* *(1773)* on practical methods and cleanliness for the prevention of puerperal sepsis and of blood poisoning from putrid wounds

Improvements in the microscope led to more definite discoveries and micro-organisms became associated with disease processes The cause of favus found by Schonlein (1839) was used by Remak (1842) to reproduce the disease Bassi (1837) from his work on disease of silkworms prophesied that microscopic organisms would be found to be the cause of human disease and similar suggestions came from many others Henle (1840) in making

* White s Treatise published in 1773 went through 5 editions was translated into French and German and was reprinted in the U.S.A in 1 93

the same prediction drew up a statement of the conditions which would have to be satisfied to provide proof of a causal relationship Similar postulates are ascribed to Robert Koch but they do not appear in concise form in his writings The first convincing discovery of microbial disease was the finding by Davaine (1850) of minute infusoria in the blood of sheep which had died of anthrax Stimulated by Pasteur he returned to this discovery in 1863 and published it finally in 1864 The disease was transferred by inoculation of healthy animals with blood containing the rod he had found and inoculation remained effective even when the blood was diluted a million times The subsequent work on anthrax by Koch (1876) and Pasteur (1877) is virtually the starting point of pathogenic bacteriology and was founded on the earlier work of Pasteur from 1857 onward

The beginnings of the science of bacteriology emanated from Pasteur s interest in fermentation which was stirred by his discovery (1848) of the selective use of dextrorotatory tartaric acid by a mold which neglected the levorotatory form His studies of lactic acid fermentation (1857) and of alcoholic fermentation (1860) led him to the necessity of disproving the hypothesis of heterogenesis (1861) in order to substantiate his demonstration of specificity of ferments The making of wine in France at that time was encountering an enormous reduction in quantity and a deterioration in quality and keeping power due partly to an *Oidium* disease of the vines and partly to *Phylloxera* This brought Pasteur to study the ' flower of wine and the ' flower of vinegar (1862) and led to his study of the making ripening and preserving of wines and beer (1863) and eventually his studies of putrefaction and anaerobiasis Diseases of silkworms next claimed his attention (1865 1869) and the procedures he instituted not only saved the industry in France but their wide adoption is still the practice it is a perfect example of the detection of infected individuals and controlling the spread of disease by isolating them The war of 1870 stimulated his studies of infected wounds and he translated his ideas of specificity of fermentations into specificity of infections From this grew Lister s work and the introduction of antiseptics with the eventual development of aseptic technic as the work of many subsequent investigators Pasteur went on to work at anthrax (1877) developing his vaccines and proving their worth (1881) then chicken cholera (1880) and swine erysipelas (1882)—to all of which his genius ensured phenomenal results in the recognition of causative agents and specific immunization Pasteur achieved greatest fame by his work on rabies Recognizing the site of infection he obtained a source of vaccine even though he could not isolate the organism and he developed a process of attenuation of the virus as well as a procedure for its application which is still used widely and seems only to be surpassed by the recent modification of the Flury strain of virus by egg passage and the use of antirabies serum Pasteur deserves to be called the father of bacteriology

Robert Koch who started his work just when Pasteur had initiated the concept of specificity of infectious disease contributed enormously and most particularly by his developments of bacteriologic technic In his studies of anthrax (1876) Koch isolated the bacillus in pure culture and established its infectivity This was the first purposive isolation of a pure culture He proceeded then to study traumatic infectious disease (1877) and developed the technic of isolation of pathogenic bacteria in pure culture from mixtures (1878 1881) so effectively that his methods are largely used today He also used the newly discovered aniline dyes to great advantage in demonstrating bacteria microscopically After Villemin (1865) had shown that tuberculosis of both man and animals could be transmitted by inoculation from man to animals and from one animal to another Koch (1882) discovered the causative organism of tuberculosis Later the differentiation into human type and bovine type was done by Theobald Smith (1896) and Rivalta (1889) and Maffucci (1890) discovered the related organism of avian tuberculosis Koch went on to the discovery of the cholera vibrio (1883) making important contributions to the knowledge of that devastating disease but his discovery of tuberculin (1890) was temporarily detracted from by the claim of its being a cure The greatest of his many contributions was the discovery of methods of isolation and study of bacteria in pure culture and the pro

cedure by which to study their infectivity

Largely as the result of the work of Pasteur and Koch the isolation and the identification of causative organisms of disease by many investigators proceeded apace Long lists could be made of pathogenic micro-organisms with the date of discovery and even longer lists of bacteria important or unimportant to other human interests These dates and authors can be found in manuals of determinative bacteriology and the exciting history of their discovery is in the original papers or special chapters of books

The isolation of the diphtheria bacillus by Letzerich and Klebs (1881) and by Klebs and Loeffler (1883 1884) and of the tetanus bacillus by Kitasato (1889) after it was seen by Nicolaier (1884), opened the way for one of the most important chapters in bacteriology the discovery of toxin and antitoxin Loeffler (1887) supposed the production of a poison to explain the results of his inoculation experiments using cultures of the diphtheria bacillus and Roux and Yersin (1888) demonstrated the toxin in filtrates of cultures This was followed by Knud Faber (1890) showing that the tetanus bacillus also secreted a toxin These various discoveries stimulated tremendous work on diphtheria resulting in the discovery of antitoxin by Behring (1890) for diphtheria and by Behring and Kitasato (1890) for tetanus The brilliant confirmation of this by Roux and Martin (1894) who first immunized horses and gave notoriety to serum therapy resulted in the spectacular drop in the mortality rate of diphtheria The first serum treatment in man was instigated by Behring and Wernicke and actually was done on Dec 25 1891 Ehrlich (1896) introduced standardization of toxin and antitoxin thereby contributing greatly to their successful use in the treatment of disease and to the knowledge of their working

In recent years through the work of Ramon (1925) active immunization with formalin treated diphtheria toxin (*Anatoxine* or *Toxoid*) has all but eliminated where it is used the incidence of diphtheria in children Before this immunization had a measure of success using toxin antitoxin mixtures which Babes (1895) had proved on guinea pigs and Behring (1913) first used on humans It was forwarded most effectively by Park (1913 1918) and controlled by the intradermal test of immunity introduced by Schick (1913) on the basis of the intracutaneous test used in animals by Roemer (1909) But the immediate influence of the early discoveries was to over emphasize the possibilities and the hopes of the humoral immunity and toxins and antitoxins were sought for everything, often in vain However, this search and interest occasioned the mixing of cultures, filtrates of cultures and immune serum, and the frequently unforeseen results introduced entirely new procedures and concepts into medicine Thus Buchner (1889) found complement (alexin) Fodor (1886) the bactericidal action of normal rabbit serum for anthrax bacilli, Nuttall (1888) the dependence of bactericidin on complement and Richard Pfeiffer (1894) showed that with cholera vibrios immunization greatly intensified bactericidal activity

Between 1870 and 1877 independent observations by Hayem Klebs von Recklinghausen Waldeyer Koch and others suggested that the leukocytes in pus in which bacteria could be seen were a suitable lodgement or a site of predilection for the microbes Metchnikoff (1883) from a study of the activity of the ameboid (*mesodermal*) cells of invertebrates and vertebrates called them phagocytes and ascribed to them a protective activity by virtue of their destruction of ingested microbes He conceived these scavenging microphages and macrophages to be the principal defense mechanism against infection and a polemic arose and lasted several years between the Cellular Defense protagonists and those for Humoral Defense Experiments to prove or disprove either hypothesis resulted in many interesting observations and the recognition of immunity reactions and responses not suspected until then Thus Denys and Leclef (1895) showed that immunization greatly increased phagocytosis and the work of Almroth Wright and his colleagues (1903) advanced knowledge of it and gave the name *opsonin* to this activity

Charrin and Roger (1889) observed that *B pyocyaneus* grown in immune rabbit serum first lost its motility and then grew in agglomerated masses contrasting with the diffuse growth of motile organisms in normal serum This was confirmed by many others for several different organisms and Bordet showed that

heating the immune erum at 56 C did not alter its effect Durham (1896) and Gruber (1896) showed that the clumping of bacteria by immune serum was specific to the kind of bacterium and Durham named the reaction *agglutination* Their work attracted keen attention and was applied immediately by Widal Grunbaum Semple and others to diagnostic tests to recognize formed antibodies in patients reacting to known cultures or to recognize unknown cultures by their reaction with known immune sera Bordet (1898) extended the reaction to specific agglutination of foreign red blood corpuscles by immune serum and Landsteiner (1900) used it for the recognition of blood groups and the investigation of the antigens of erythrocytes a knowledge now of much importance in blood transfusion methods Castellani (1902) devised the *absorption of agglutinin* test which ultimately led to the methods of antigenic analysis by purified absorbed sera and to the present trend to definition of bacterial species by their antigenic structure as illustrated by the genus *Salmonella*

In investigating agglutination of erythrocytes by immune serum Bordet (1898 1899) observed the lysis of the cells and established the relation of this lytic reaction to complement showing that in the absence of complement simple agglutination occurred He demonstrated the strict specificity of the reaction and showed that the complement was removed from the system in the process Bordet and Gengou (1901) and Gengou (1902) used this immune hemolytic system as a delicate means of detecting the presence or the absence of free complement They showed that any immunity reaction taking place in the presence of complement removed or fixed the complement and thus they devised a most delicate test for the detection of interaction between antibody and antigen This prompted many workers to apply the test to all sorts of purposes the most widely known of these is the application of it by Wassermann Neisser and Bruck (1906) to the erodiagnosis of syphilis

When mixing the filtrate of a cholera culture with its corresponding antiserum Rudolph Kraus (1897) observed that a precipitate formed rapidly in the mixture and soon aggregated into flocculi which settled to the bottom of the tube Identical results were obtained with typhoid and plague bacilli but the reaction was strictly specific to the kind of bacterium This observation was abundantly confirmed and quickly extended by Tchistovitch (1899) to eel serum by Myers (1900) to egg albumen and by others to proteins of every source and to bacterial polysaccharides It quickly had diagnostic applications in disease and Nuttall (1902 1904) applied it to indicating phylogenic relationship between mammalian species It was applied to the identification of origin of blood stains (human sheep etc) the sources of blood sucking insect meals and of the adulteration of foods It has had a most important application in the investigation of the mechanism of immunity reactions because it allows close quantitative determinations

Out of this welter of new discoveries one arising out of another and pointing in all directions fiercely contended polemics arose and conflicting theories were elaborated to explain the situation The theory of immunity propounded by Ehrlich (1896 1899 1903 1910) and known as the side chain theory primarily depended on the assumption of chemical affinity between antibody and antigen with adjuvant factors added for special purposes It did much to co ordinate ideas and above all it afforded workers a terminology by means of which they could express themselves to be clearly understood If for no other reason than this Ehrlich s theory was a tremendous factor in the advances made in immunology in its early days Ehrlich also made lasting contributions to experimental immunology to chemotherapy and to the standardization (1896) of diphtheria antitoxin He distinguished active and passive immunity and characterized toxin However some observations were difficult to explain without making extensive new assumptions and Bordet (1898 and 1909) introduced a physicochemical theory depending on a specific union of antibody and antigen with the possibility of a chemical basis determining a specific adsorption followed by a nonspecific action of electrolytes and a shift of electrical charge to result in the final manifestation of the reaction This largely replacing Ehrlich s hypothesis stimulated a lot of exacting work over a period of years and became elaborated to the view that antibody is pecifically ad

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lich observed that cane sugar milk sugar glycerine and even cellulose are assimilated These were still the days when Cohn and others wrote with serious need on distinguishing pseudobacteria In the literature from 1900 onward the descriptions of action on increasing numbers of sugars are common they are tabulated in Matsuschita s book (1901) and are prominent in other early text books Special tubes for the appreciation of these fermentations were recommended by Theobald Smith and by Durham and are still in use

As methods for the isolation and the identification of bacteria improved interest developed in their natural distribution and in the transmission of infection to cause disease Case to-case transmission by contact and by fomites was an expansion of the ideas of early speculative times and was substantiated by the instances of wound infection puerperal sepsis smallpox etc The extension of it to contamination of the environment clothing and personal articles followed naturally It was accentuated by the doctrine of surgical fever and the phlogogenic and pyrogenic properties of pus started by Billroth (1860) and by the finding by Coze and Feltz (1866 1872) of infuzoria (either motile or in chains name coined by O F Muller 1773) in the blood and the fluids of animals injected with putrid substances which increased in lethal power in successive injections Impetus which carried final conviction was given to the germ theory of disease by Klebs (1870 1873) investigating gunshot wounds with consequent septicemia and pyemia and the publication by Koch (1878) of his work on the *Aetiology of Traumatic Infective Disease* Koch s technical methods allowed Ogston (1880-1883) to associate uppuration and inflammation spreading to septicemia and pyemia with micrococci he named *Staphylococcus* and with Billroth s *Streptococcus* and led him to produce lesions in animals with cultures in eggs

The recognition of contamination of water supplies and food through improper disposal of excrement brought about a new phase in the understanding of disease and led to legislation and control based upon bacteriologic knowledge Snow (1849) first correlated epidemic cholera with contamination of water supplies and added confirmatory evidence during the epidemic of 1854 Although Budd (1856) still anticipating bacteriology pointed out that typhoid is transmitted by the excreta of the patient, water borne typhoid was not recognized until 1872, by Hagler in Switzerland and milk was incriminated by Radcliffe and Power (1873) in the St Marylebone outbreak of enteric fever due to washing of utensils with polluted water Flugge says in his textbook (1886) that pathogenic bacteria have never as yet been demonstrated with absolute certainty in water but at that time the viability of typhoid bacilli and cholera vibrios in water was investigated by Bolton (1886) and a little later by Frankland Chantemesse and others It seems that the first isolation of typhoid bacilli from water was by Remlinger and Schneider (1896) and in the soil of infected barracks by Tryde and Salmonsen (1885) the first attempt at the sterilization of water mains was in the Maidstone epidemic of 1897 on the recommendation of Sims Woodhead using large quantities of chloride of lime The finding of typhoid bacilli in naturally contaminated water is still a difficult procedure and the recognition by Theobald Smith (1892) of the necessity of estimating the coliforms in water as an indication of fecal contamination proved to be an important step The tracing of many notable outbreaks of typhoid paratyphoid cholera food poisoning diarrhea dysentery scarlet fever septic throats infectious jaundice etc to contamination of water milk and prepared foods has led to much special knowledge and to regulation and control Sewage disposal for the same reason became principally concerned with elimination of contamination by pathogenic organisms and only secondarily with esthetic and economic considerations

Case-to case transmission even by indirect means of water food and fomites did not account for the incidence and the distribution of many cases in epidemic cerebrospinal meningitis and failed to explain satisfactorily the source of some outbreaks of typhoid The persistence of diphtheria bacilli in completely recovered cases was demonstrated by Roux by Yersin and by Loeffler (1890) but their importance was not fully recognized Albrecht and Ghon (1901) proved the presence of the meningococcus in the nasopharynx a finding

sorbed forming a partial or complete surface film This theory too fails to fit all situations discoverable in immunity Nor were these situations explained by the theory enunciated by Arrhenius and Madsen (1907) invoking a reversible reaction analogous to that of weak acids and bases and supposing the possibility of coexistence of free antibody free antigen and antigen antibody complex The newest theory is that of Heidelberger (1935) This developed out of an extension of the work of Dean and Webb (1926) which showed antibody and antigen to react in terms of relative proportions independent of concentration At the point of reference of optimal proportions there is no free antibody and no free antigen this they regard as the *equivalent ratio* Heidelberger introduced more delicate methods of determining quantitative reaction and measured and defined the action taking place more exactly Heidelberger considers the reaction as the resultant of competing bimolecular reactions and the composition of the complex depends on the relative proportions in which the components meet rather than the concentration The situation can be expressed by *mass action* equations This theory and Heidelberger s technic have stimulated most important work on a variety of problems including antigenic structure and specificity Thus there is a return to the importance of chemical constitution as the determining factor in immunity

The application of agglutination and absorption of agglutinin reactions to the identification of bacteria resulted in the recognition of multiple antigenic components in the cells This found expression in the definition of *types* within the species the first of which was related by Gordon and Murray (1915) to the meningococcus and others have since applied it to many other species notably the pneumococcus and the streptococcus The definition of *type* and of *group antigens* in Pneumococcus by Avery and in Streptococcus by Lancefield (1928 1933) proved to be of great value and also indicated a relation between antigenic significance and chemical character The importance of types was confined at first to directing specific serum therapy and the tracing of epidemiologic information both of which led to an expansion of interest and an emphasis on immunologic character in the exact identification of bacteria The method was refined by the purification of sera by specific absorption to allow prompt and certain identification of antigenic components When this method was applied intensively, while taking into account the flagellar and somatic antigens first discovered by Theobald Smith and Reagh (1903) the rough and the smooth characters described by Arkwright (1921) the H" and the 'O conditions demonstrated by Weil and Felix (1920) and the diphasic variation discovered by Andrewes (1922), the complex distribution of the antigenic components in the variants of the species caused much confusion Order was introduced in the case of *Salmonella* by Bruce White (1934) and by Kauffmann (1934) so that by their work and that of many others strains in that genus can be defined by the antigens they exhibit

Nevertheless although there is an orderly arrangement by this method it has not yet allowed a satisfactory immunologic definition of bacterial genera and species

Confusion still besets the immunologic definition of many other kinds of bacteria others have not yet been subjected to it so the accustomed criteria of morphology growth characters and requirements use of hydrolysable or fermentable substances loosely called sugars and the recognition of various by products of metabolism still have to be relied on for differentiation and identification of bacteria These culture methods which gave prodigious results were made possible by four very simple technics the introduction of the cotton plug by Schroder and von Dusch (1854) the sterilization of media by heat devised by Pasteur (1877) in part an application of the marmite of Papin (1681) and Koch (1881) the use of dyes by Weigert (1871 1875) and elaborated by Koch (1877) and Ehrlich (1879) and the development of solid culture media by Koch (1881) The authorship of the use of sugars is obscure but it should rank almost as highly as the above Sternberg (1884) says that Pasteur took less account of structural characters than did Cohn Nageli Dujardin and others and suggested that cultural requirements and special kinds of fermentation be relied on At the same time Sternberg questions the value of 'these species purely physiological' Before 1884 Mitscher

lich observed that cane sugar milk sugar glycerine and even cellulose are assimilated These were still the days when Cohn and others wrote with serious need on distinguishing pseudobacteria ' In the literature from 1900 onward the descriptions of action on increasing numbers of sugars are common they are tabulated in Matsuschita s book (1901) and are prominent in other early text books Special tubes for the appreciation of these fermentations were recommended by Theobald Smith and by Durham and are still in use

As methods for the isolation and the identification of bacteria improved interest developed in their natural distribution and in the transmission of infection to cause disease Case to case transmission by contact and by fomites was an expansion of the ideas of early speculative times and was substantiated by the instances of wound infection puerperal sepsis smallpox etc The extension of it to contamination of the environment clothing and personal articles followed naturally It was accentuated by the doctrine of surgical fever and the phlogogenic and pyrogenic properties of pus started by Billroth (1860) and by the finding by Coze and Feltz (1866 1872) of infusoria (either motile or in chains name coined by O F Muller 1773) in the blood and the fluids of animals injected with putrid substances which increased in lethal power in successive injections Impetus which carried final conviction was given to the germ theory of disease by Klebs (1870 1873) investigating gunshot wounds with consequent septicemia and pyemia and the publication by Koch (1878) of his work on the *Aetiology of Traumatic Infective Disease* Koch s technical methods allowed Ogston (1880 1883) to associate suppuration and inflammation spreading to septicemia and pyemia with micrococci he named *Staphylococcus* and with Billroth s *Streptococcus* and led him to produce lesions in animals with cultures in eggs

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notably expanded by von Lingelsheim (1906) to culminate eventually in the full elucidation of the role of healthy carriers by Gordon, Flack Glover (1915 1917) and others The investigation of the typhoid carrier dates from the statement by Koch (1902) that the typhoid patient or convalescent who happened to harbor the specific germ was the source of further infections The bacteriologic examination of convalescents by Frosch Drigalski, Donets (1903 1904), soon followed by others proved the existence of fecal and urinary carriers At the same time the existence of *atypical ambulatory cases of enteric fever* was recognized and an identical situation was soon found to prevail for paratyphoid and acute food poisoning (*Salmonella*) So the possibility of the carrier condition was investigated extensively and obtained a prominent position as a source of preservation and dissemination of disease

Among the diseases of antiquity rabies anthrax and tuberculosis were associated with animals and the realization that they can be transmitted to man was delayed It was known that rabies was transmitted by the bite of a mad dog but man was thought to be immune until Celsus in the 1st century A D gave the name hydrophobia to the human disease which was better described in the 2nd century by Celius Aurelianus The paralytic form in man was recognized by van Swieten (1770) and Zinke (1804) demonstrated the infectivity of dog saliva to rabbits dogs and chickens Gruner (*1813*) recommended diagnostic inoculation of saliva from suspect dogs into test animals and Magendie and Breschet (1821) identified human rabies with the animal disease by infecting a dog with saliva from a human case This opened the way for Pasteur (1880) to study the transmission and the control of rabies

Devastating outbreaks of what appears to be anthrax of animals are found in the earliest writings The disease was recognized in man only toward the end of the 16th century and the black bain was at times a scourge Fournier (1769) described it as a disease of man and animals and about this time Morgani, because of his opinions on infectious diseases would not risk opening the chest of a wool comber suspected to have died of pulmonary anthrax Human anthrax became recognized as a hazard of hide and hair handlers and infection from shaving brush bristles imported from Siberia and China was proved (1915) by Elworthy

Tuberculous lesions have been found in Egyptian mummies The presence of tuberculous lesions in domestic animals gave rise to laws prohibiting their use as food The lesions were subject to much confusion until the contagiousness of the disease was suspected by Ruhling (1774) and animal tuberculosis was thought by Huzard (1790) to be identical with the human disease though this view was not generally held Koch s discovery of the tubercle bacillus (1882) led to the finding of it in cows milk by St Friis (1893) which was quickly confirmed and followed by *Theobald Smith (1896) who differentiated the* human and the bovine varieties The finding of the bovine tubercle bacillus in human cases was reported by Ravenel (1901) and its relation to tuberculosis especially of children was widely investigated thereafter by Park and Krumwiede (1910) and by A S Griffith (1914 onward) who also first found it in cases of phthisis It is also the bovine variety which Calmette and Guerin (1908) modified by growth in bile to produce the B C G strain used for vaccination (1921 onward) but protection has also been afforded by immunization with the vole bacillus of Wells (*1937*) a species with naturally restricted infectivity The infectiousness of cattle tuberculosis to man urged the pasteurization of milk and the *tuberculin testing of dairy herds together with* Government eradication schemes Avian tuberculosis proved to be more an economic problem in poultry and swine only a few cases have been reported in man

Malta fever long known in man was brought into the category of animal diseases transmissible to man by the British Commission (1905 1907) by incriminating goats milk the disease has also been found in cows sheep mules and horses Contagious abortion of cattle was suspected as infective for man after Schroeder and Cotten (1911) found *Brucella abortus in milk*, and Larson and Sedgwick (1913) found antibodies in children but the organism was first isolated by Duncan (1925) from a human case *Brucella suis* is found in swine horse fowl and dog it is chiefly American in distribution and

Keefer (1924) isolated it first from a human case

Plague early made itself evident as a human disease but it is an open question whether the Philistine offering of mice and of emerods made of gold indicate recognition of its rodent origin An 800 year old sacred Hindu book *Bhagavat Purana* describes human and rat plague and recommends leaving houses where dead rats are found Simond and Hankin (1898) suggested that plague was carried by fleas and this was supported by Ashburton Thompson (1900) and Blackmore (1902) who did much to show the relation between rat epizootics and human plague but the definitive association of plague with rodents and fleas dates to the report of the Indian Plague Research Commission (1906 1908) There was great skepticism of incriminating rats and Lowson (1894) at the time Kitasato saw the plague bacillus and Yersin cultured and described it writing of the Hongkong outbreak said too much was being made of the infection of rats Wu Lien teh (1923) states that references to rat mortality in the old European plague records are few and far between and lose much of their value by the inclusion of other animal species which we know now to be insusceptible However with the recognized importance of rodents other than rats it is strange that Wu Lien teh could find no earlier reference to tarbagan plague than 1895 which lends significance to the observation that in California where plague first appeared in 1900 the infection in wild rodents was not proved until 1908

Tularemia primarily a septicemic disease of rodents was first proved in a human case by Vail Wherry and Lamb (1914) and subsequently recognized and named by Francis (1920) Besides being acquired by the handling of infected animals (mainly jackrabbits) it is transmitted by blood sucking flies and ticks An interesting historical feature of this disease is that the recognition of its actual geographic distribution was so slow and bacteriology was so far advanced at the time of its discovery

There are other important animal sources of human infection among which is food poisoning due to members of the genus *Salmonella* Most outbreaks of this have been due to prepared foods of animal flesh and in Europe it has been associated with the slaughter of sick animals used for human food The historical instance was the discovery of *Salmonella enteritidis* by Gärtner (1888), following the illness of 57 people who had eaten the meat of a sick cow Sheep have been an especially common source of aertrycke infections in man and at the present time dried egg powder has been shown to yield a number of different salmonellas

With such discoveries as those of Manson (1877) on the transmission of *Filaria bancrofti* by the bite of *Culex fatigans* of Bruce (1895) proving the transmission of Ngana and later of Ross work on *Proteosoma* at a time when the bacteriologic cause of disease was itself a very exciting novelty it is not surprising that the role of insects has been invoked as a possibility in bacterial infection from time to time However transmission of bacterial disease by blood sucking arthropods has relatively few examples Plague for example is transmitted by various species of rodent fleas especially by the oriental rat flea (*Xenopsylla cheopis*) to man the relapsing fevers by the louse (*Pediculus humanus*) and by ticks (*Ornithodorus* of different species) varying strictly according to the species of spirochete concerned tularemia by deer fly (*Chrysops discalis*) ticks (*Dermacentor andersoni*) rodent lice (*Haemodipsus ventricosus* and *Polyplax serratus*) a squirrel flea (*Ceratophillus acutus*) and the stable fly (*Stomoxys calcitrans*) which has been blamed without justification for many crimes Cases have occurred of possible transmission of anthrax and other diseases by the soiled proboscis of biting flies but these have the character of accident

The mention of flies and disease usually conjures up a vision of that teasing and restless insect with unpleasant habits the housefly (*Musca domestica*) and there is no doubt of its complicity in the spread of typhoid dysentery cholera and food poisoning salmonellas It has been shown that flies are urgently attracted to fecal matter and decaying substances in which they lay their eggs or on which they feed They have the habit of emptying their crop wherever they may be either to take up the material again or to leave it to eat afresh and they defecate freely while feeding The vomit spots and fecal spots

of flies are found everywhere, and the circumstances which favor spread of disease by flies vary partly with the climate but most with the prevailing sanitary conditions This information was accumulated by intricate and difficult experiment and observation Sydenham (1666) observed that an abundance of houseflies in the summer was succeeded by an unhealthy autumn and Leidy (1864) attributed the spread of hospital gangrene to flies Howard (1895) began the study of the bionomics of the housefly and proposed (1911) that it be called typhoid fly though admitting that the phrase was an overemphasis Hewitt (1907 1912) made detailed studies of the anatomy of flies and believed that the housefly plays an important part in the dissemination of disease when the necessary conditions are present ' To Howard and Hewitt great credit is due for the work they did mainly based on epidemiologic evidence as well as the work they stimulated A long series of experiments was carried out by Graham Smith (1910 onward) on the distribution of bacteria by nonbiting flies he very carefully studied their feeding mechanisms the functions of the crop and the proventriculus the process of regurgitation and defecation as well as the habits of flies especially before and after feeding The range of flight of houseflies was first studied by Arnold (1907) and extended by Copeman Howlett and Merriman (1911) Hewitt (1912) and others giving the greatest range observed as 700 yards of actual flight Sandwith (1904) described the habits of flies conducive to spread of disease especially of ophthalmia in Egypt and Bancroft (1769) speculated on the transmission of yaws by flies Nichols (1912) believed a small fly (*Oscinus pallipes*) to be responsible for inoculating surface injuries and causing the majority of cases of yaws in the West Indies he also demonstrated that wild flies carry fecal contamination Hamilton (1903) was the first to isolate typhoid bacilli from wild flies caught in the vicinity of cases of typhoid in Chicago Ficker (1903) did the same in Leipzig and similar observations were soon made by many others on various kinds of organisms and different species of flies Extensive investigations were carried out by many of the above mentioned observers and by others on the distribution and the duration of the bacteria on and in the flies, and epidemiologic data of all kinds was collected However though abounding in interest this important phase of bacteriology is still far from complete

The need to grow bacteria in pure culture on prepared media, with the limitations set by bacterial morphology as revealed by microscopy, developed the method of differentiation by their physiologic processes This led naturally to the discovery of special growth requirements and biochemical activities and so by stages to the fruitful study of enzymes on the one hand and to the use the production and the estimation of vitamins on the other These are active lines of investigation which have not yet spent the impetus of the interest they inspire of themselves nor have they outrun the promise of explaining the action of selective disinfectants of the pathologic processes in some diseases and of benefits conferred by bacteria on other forms of life

John Pringle read several papers to the Royal Society of London between 1750 and 1752 on Experiments upon septic and antiseptic substances introducing the first use of the word 'antiseptic ' Lister's "ideal antiseptic seemed to be on the verge of realization with the discovery of the sulfonamides Landmarks in the field are the pioneer work of Ehrlich (1904 1915) on chemotherapy especially in syphilis (1910) the clinical use of prontosil by Foerster (1933) and the communications by Domagk (1935) Colebrook and Kenny (1936) established the remarkable action of prontosil in streptococcus infections This was followed (1938 1941) by the introduction of sulfanilamide sulfapyridine sulfathiazole sulfadiazine etc , with safer and wider applications to gram positive organisms Very quickly numerous synthetic products of the same general chemical nature were put on trial and a frantic search was rampant for products acting on a wider range of bacteria or regionally limited to intestinal infections and, above all with less toxicity to man The benefits that these agents introduced in the treatment of war wounds and the freedom their use gave to the surgeon had a profound effect almost seeming to reintroduce antiseptic surgery ' The urgency of war increased the volume and the rate of investigation to levels which never could have been realized

in times of peace The experiences in production and the realization of chemotherapeutic possibilities thus gained was instantly transferred to the astounding production of penicillin when its possibilities and advantages were demonstrated

Incidental observations on synergy and antagonism between bacteria are scattered widely through the literature from quite early times but usually there is little indication of more than a passing interest thus Garre (1887) and Freudenreich (1888) showed that media which had supported growth of one kind of bacterium might become antiseptic to another On the basis of antagonism Colebrook (1916) attempted to free meningococcus carriers by infecting their nasopharynx with pneumococcus Rettger and Speary (1912) showed the bactericidal properties of egg white Fleming (1921) made extensive studies of lysozyme in tears and in the secretions of mucous membranes A sustained interest in this type of action led Fleming (1929) to the discovery of penicillin which was developed by Florey (1941) to startling clinical applications Dubos and Avery (1930) extracted from a cranberry bog bacillus an enzyme which digests off the capsule of pneumococcus Type III rendering it avirulent With this enzyme they could protect mice from infection and later they found specific enzymes to act on the capsule of other pneumococcus types The efficacy of penicillin and its innocuity to man has led to the persisting stupendous world wide search for antibiotics from every possible source A number have been described but only streptomycin discovered by Waksman (1940-1945) has so far proved to be of outstanding importance Tyrothricin discovered by Dubos (1940) has only limited applications The complexity of the therapeutic applications and the need for proper control of antibiotics has if any thing enhanced the importance of the bacteriology laboratory Furthermore this actively growing phase of bacteriology has a very interesting bearing on the study of bacterial variation the importance of which is still to be fully appreciated including the development of drug fast strains

Comparisons of substrates and products of enzyme activity between host cells and parasites have revealed competitive antagonisms by analogues of metabolites but the design to discover thereby therapeutically ideal anti metabolites has not yet proved to be productive Nonetheless a diversity of detailed knowledge has accumulated with appreciation of new intricacies in the processes of infection and of limitations due to adjustments of quantitative rather than qualitative relations There has been shown a delicate balance limiting control of abnormal cells or damage to normal cellular activity by analogues of metabolites complicated by development of resistance and even dependence this is exhibited not only by bacteria but also by experimental animals and patients Important influences of metal cations on microbial activities have been revealed by studies of the chelating action of antibiotics and other antimicrobial agents though it is remarked that most chelating agents are not antimicrobial The selective activity of identified cations on various kinds of bacteria indicate important physiologic differences between them but the key enzyme systems affected by antimicrobial compounds have not been revealed by studies thus far reported' Beyond the finding of diaminopimelic acid and hydroxy methyl cytosine no distinctive biochemical character of bacteria and viruses has been revealed Thus it proves to be difficult to segregate problems as purely bacteriologic, biochemical physiologic or clinical in considering infectious disease

In recent years there has been an awakened interest in mediators of infection there has been widespread inquiry into the influence of constitutional and hereditary characters nutritional and metabolic variations hormonal disturbances and irradiations in relation to establishment and progress of infections Mucins polysaccharides and new found proteins in plasma cells and tissues promise revelations in terms of susceptibility lethality localization and dispersion of bacterial infections and concomitantly according to their nature their influence in permeability of capillaries and membranes as well as their role in phagocytosis Thus the subject is being invigorated by a new idea which is aptly expressed as a *biochemical lesion*

Because of their selectiveness their precise indications and their availability and rapid generation bacteria became used extensively

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vide the solution of the mystery of the nature of viruses Certainly the famous lines of Swift inspired by Leeuwenhoek finding a mite on the pupa of a flea thus become even more appropriate

SUGGESTED ADDITIONAL READING

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Bulloch W 1938 The History of Bacteriology New York Oxford

Dubos R J 1950 Louis Pasteur Free Lance of Science Boston Little

Ford W W 1939 Bacteriology Clio Medica New York Hoeber

Long E R 1928 A History of Pathology London Baillière Tindall & Cox

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for the study of enzymes and intracellular physiology for bio-assays in great variety and for experiments in genetics But with the development of electron microscopy and the cutting of ultrathin sections advances have been made in the knowledge of the structure of the bacterial cell itself this besides its intrinsic worth and the demonstration of peculiarities which promise taxonomic advantages is not without significance to other biologic sciences The indications of structural and behavior distinctions between the bacterial nucleus and those of animals and plants seem likely to require a new general theory of the processes of this organelle Although the exact structure of the bacterial nucleus and its reproductive behavior have not yet been described in a manner that everyone accepts transfer of genetic determinants has been recognized by observing hereditable physiologic biochemical and immunologic activities Certain instances appear to resemble gene recombinations with linkage in some instances and a suggestion of mating and even hybridization It is still unknown whether these experimental sexual recombinations occur in nature and whether evolutionary instability in the strains is required The perhaps more mysterious hereditable transformation of the serotype of pneumococcus strains by treatment of the receptor cells (preferably in the R phase) with a desoxyribonucleic acid extract of the donor cells results in the receptor acquiring a new pattern of synthesis of the immunologically type specific capsular polysaccharide together with the corresponding transformation of its active nucleic acid This has the appearance of a soluble genetic material which cells can be made to absorb without their having an absolute need for doing so

It has also been shown beyond any doubt that lysogenic bacteriophage (virus) can carry a genetic factor acquired from a donor cell in which it was grown and substitute it appropriately by replacement in the genetic structure of a receptor bacterial cell The receptor cell reproduces with the characters it has acquired instead of its original form and these involve structure (flagella) specific antigenicity and nutritional functions This happening was discovered by N D Zinder and J Lederberg (1952) and named *transduction*, it evidently differs from gene recombination, but how it is accomplished and whether it occurs in nature is unknown ' Bacteriological studies may therefore be expected to contribute even more in the future than they have in the past, and that not confined to the processes of infectious diseases But their significance will largely depend on integration with studies in other fields "

Haffkine (1896) described the self sterilizing power of the water of the Jumna River, which was lost when the water was boiled but the possibilities of this observation were not realized until the discoveries of Twort (1915) and D Herelle (1917) were appreciated Twort described a lytic phenomenon and a vitreous appearance of cultures of staphylococcus derived from vaccinia lymph The transforming agency could be transmitted to normal living cultures but failed to act on dead cultures the active principle was filterable and somewhat heat resistant He formulated the several possibilities which might explain the phenomenon but did not insist on any one of them D Herelle rediscovered the same phenomenon when working with the Shiga dysentery bacillus extended the investigations considerably further to other bacteria and even applied it to therapeutic purposes in man He maintained that the effect was wrought by a living parasite of the bacterial cell and called it *bacteriophage* The further history of bacteriophage belongs to the viruses but their remarkable specificity to host requirements has been used to recognize race and type differences within bacterial species by Evans (1934 1940) with streptococcus by Craigie (1938 1942) with the typhoid bacillus by Keogh (1938) with the diphtheria bacilli and by Fisk (1942) with staphylococcus The phage typing of the typhoid bacillus has proved to be of epidemiologic importance in the hands of Helmer Kerr Dolman and Ranta (1939) and of Foley (1942) in tracing the source of outbreaks or individual cases It has too a theoretical importance because the selectivity of the typhoid bacteriophage has been associated with the Vi antigen and diphtheria toxin synthesis which thus links it to problems of pathogenicity and protective immunization Furthermore this most strange parasitism may well pro

making immense contributions to the improvement of health But it is also increasingly apparent that without benefit of drugs or vaccines the majority of human beings as well as of animals and plants commonly reach a state of equilibrium with even the most virulent pathogens In the normal course of events all sorts of micro-organisms become established and persist in the tissues without interfering with the normal physiologic processes of their hosts—a condition that one might call peaceful coexistence The very fact that infection need not result in disease and indeed rarely does suggests that aggressive warfare against pathogens may not be the only profitable approach of medical microbiology to the maintenance of health it points to the possibility that technics could be developed for rendering the body capable of converting parasitism into commensalism Admittedly this theoretical view is at the present time a counsel of perfection of little practical import knowledge of the fact that a state of peaceful coexistence can and often does exist between parasite and host has not led as yet to any useful policy of disease control Nevertheless it seems worthwhile to review briefly in this chapter some of the facts that have a bearing on the outcome of the contact between micro-organisms and their hosts

The virulence of the micro organism naturally plays a decisive part in influencing the course of any infectious process For the purpose of the present analysis virulence may be regarded as the composite expression of two different classes of effects On the one hand the virulent micro-organism must possess attributes—to be referred to here collectively as infectivity—which render it capable of establishing a foothold and of multiplying in one or several organs On the other hand it must also be endowed with pathogenicity i e with the power to cause pathologic changes by altering tissue components or interfering with their normal functions One might assume at first sight that micro-organisms possessing both infectivity and pathogenicity would inevitably cause disease when introduced into a receptive individual However as already mentioned the observed fact is that most normal and healthy human beings as well as most other living things constantly harbor in their tissues a variety of viruses rickettsia bacteria protozoa and metazoa which are infective and pathogenic For example the majority of adults all over the world have been infected at some time with poliomyelitis viruses and with tubercle bacilli yet only a small percentage of those infected develop paralytic poliomyelitis or clinical tuberculosis It is certain that among men and animals the ability to prevent microbial agents from expressing their pathogenicity is much more common than the ability to ward off infection

The manner in which an individual responds to a given infectious agent is conditioned by his immunologic past his constitution and his environment Among these conditioning factors the state of specific acquired immunity is the one which has received the most attention during the bacteriologic era and concerning which the most knowledge is available This aspect of the problem need not be further discussed here except to emphasize again that acquired immunity is often more effective in controlling the manifestations of disease than in preventing the establishment of infection Whether humoral or cellular whether directed against the cellular constituents or the toxic products of the pathogen acquired immunity often fails to prevent the latter from gaining a foothold in the tissues or to eradicate it once infection has taken place For example a level of specific humoral antibodies sufficient to decrease greatly the incidence of paralytic poliomyelitis does not necessarily protect the exposed individual from becoming infected with polio viruses nor does it prevent the latter from multiplying in the intestinal tract for a while at least Similarly the cellular type of immunity elicited by BCG vaccination protects the vaccinated person against the acute miliary or meningeal forms of tuberculosis but does not prevent the virulent tubercle bacilli from becoming established in the body or even from producing small lesions The fact that all kinds of pathogens can persist in the tissues following either subclinical infection or recovery from overt disease is so general in occurrence that it can be regarded as the rule rather than the exception And yet there is no doubt that persistence of any given pathogen in some parts of the body results in increased resistance to this pathogen —a state that has been referred to as premunition

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2

The Evolution and the Ecology of Microbial Diseases

INFECTION* VERSUS DISEASE

The mechanisms through which microbial agents reach their potential victims and elicit pathologic reactions are known in their broad outline But this knowledge has not yet been reconciled with the fact—now well established—that extremely virulent pathogens are often present in the tissues of normal individuals who exhibit neither signs nor symptoms of disease Today the most puzzling problem of medical microbiology is no longer How do micro organisms cause disease? but rather Why do pathogens so often fail to cause disease after they have become established in the tissues? Curiously enough this question is *rarely asked* and even more rarely submitted to experimental analysis One of the reasons for this neglect may be found in the biologic philosophy on which the doctrine of medical microbiology is based namely the assumption that parasitism and not commensalism is the normal expression of interrelationships between the microbial world and other living things

The germ theory of disease was formulated during the second half of the 19th century at a time when biologic and social philosophy was under the spell of Darwin s and Spencer s views on survival of the fittest, and of Huxley s oratory depicting Nature as red in tooth and claws Struggle between living things was then regarded as the iron rule of life The ability of an organism to overcome its competitors and to destroy its enemies was deemed an essential condition of its biologic success Although this 19th century concept of the law of the jungle is becoming unfashionable in biologic and even in sociologic circles it still dominates the science and the practice of medical microbiology and is reflected in the language used to describe the phenomena of infection The microbe is an *aggressor* which *invades* the tissues the body *mobilizes* its *defenses* the physician is a disease *fighter* whose goal is to achieve the *conquest* of infection This attitude is a far cry from the Hippocratic view that health is universal harmony and that the role of the physician is to restore equilibrium between the various components of the body and the whole of Nature

Aggressive warfare against microbes has been the orthodox approach of medical microbiology to the problems of disease and it is obvious that this policy has made and is still

* In the present essay I have used the word infection to refer to the presence of micro organisms in the tissues—whether or not it results in detectable pathologic effects This definition is at variance with that given in Dorland s *Medical Dictionary* Invasion of the tissues of the body by pathogenic organisms in such a way that injury followed by reactive phenomena results However it correspond to widespread usage among students of infectious diseases as for example in the expressions latent infections dormant infections and inapparent infections

warped the judgment of the European visitors there is no reason to doubt the validity of their unanimous opinion concerning the health and the vigor of the Polynesian people at that time The Europeans saw in all the Islands robust and happy men and women obviously well adapted to their environment including their local pathogens Yet disease became rampant among the islanders within a short time after these early explorations and the Polynesian population soon began to fall From approximately 300 000 at the time of Cook s first visit in 1778 the population of native Hawaiians had fallen to 37 000 in 1860 During the same period the population of the New Hebrides was reduced to one tenth of its original size

There is no doubt that this holocaust was brought about largely through the venereal diseases tuberculosis scarlet fever measles and other infectious disorders that the Polynesian contracted during their short contacts with the Europeans When measles was introduced into Hawaii practically the whole population went down with the disease and many thousands died Epidemics of measles pertussis and influenza occurred again in 1848, and every child born that year died when smallpox struck in 1853 there were over 9 000 cases with 6 000 deaths out of a population of 70 000 During his second and third visits to the South Pacific Cook himself was much disturbed at the thought that his sailors were responsible for having introduced venereal diseases among the Polynesians in Tahiti however he found solace in the belief that the initial guilt was to be placed exclusively on Bougainville s French crew

Many other epidemic outbreaks of disease could be selected to illustrate the destructiveness that viruses and bacteria often exhibit when first introduced into a population In Europe one could quote the fantastic mortality caused by plague during the Justinian era and again during the Renaissance The sweating sickness that suddenly struck certain areas of England in Tudor times also proved to be disastrous as can be judged from contemporary accounts and from John Caius famous treatise A Boke or Counseill Against the Disease Commonly Called the Sweate or Sweatyng Sicknesse

Several enlightening examples have occurred recently A typical illustration of the virulence of a disease in a new population was the outbreak of measles in 1952 among the Eskimos of the Canadian Arctic the attack rate in this instance was over 99 per cent including all ages and the mortality rate reached up to 7 per cent at Ungava Bay An outbreak of poliomyelitis among a group of Eskimos of the Hudson Bay in 1949 exhibited the same pattern Fourteen per cent of the population died and over 40 per cent developed paralytic disease In this case again all age groups were involved the lowest clinical attack rate being in the infants Still another example has been made familiar by the writings of Albert Schweitzer Sleeping sickness (African trypanosomiasis) is a new disease in the Ogowe region of Equatorial Africa It was introduced some 30 years ago by carriers that came with the Europeans from Loango where it apparently has existed from time immemorial The disease proved to be terribly destructive in its new territory carrying off one third of the population in the course of a few years In Uganda it killed 200 000 persons out of 300 000 in 6 years Of the 2 000 inhabitants in a village of the Upper Ogowe only 500 surviving after 2 years of the epidemic

The examples cited—and these are only a few among many—leave no doubt that whole populations can be decimated by pathogens with which they have had little contact in the past On the other hand it is becoming apparent that the diseases introduced by the white man in the 18th and the 19th centuries no longer exhibit among the Amerindians the Polynesians and other primitive people the very acute course with a rapidly fatal outcome which was uniformly observed in the past These people have developed or are developing a manner of biologic response to infection which is similar to that seen in the Western World under normal conditions The high mortality rates caused by the plague bacillus or by the yellow fever virus among Europeans probably can serve as an analogy to the type of virulence that the tubercle bacillus or the measles virus had for the Polynesians or the Amerindians 2 centuries ago Tuberculosis and measles are still important causes of disease among these people today but rarely give rise to catastrophic epidemics in the areas where

HISTORICAL CHANGES IN THE VIRULENCE OF MICROBIAL DISEASES

Resistance to microbial diseases is conditioned not only by the state of acquired immunity but also and even more perhaps by other attributes of the individual which are independent of any earlier contact that he may have had with the pathogen under consideration We shall consider in turn first those attributes which have a genetic basis then the factors of the external and the internal environments which vary from time to time and from one situation to another and thereby exert transient and reversible effects on susceptibility and resistance

That innate hereditary differences exist among human beings with regard to their resistance to infection has long been taken for granted but is difficult to prove Familial susceptibility to tuberculosis and the fact that possession of the sickle cell trait confers resistance to malaria are two instances in which the role of hereditary factors in infection has been well documented Furthermore there is little doubt that the different races and ethnic groups of men differ greatly in their response to most microbial agents but rigid proof that the differences are hereditary has been rendered difficult by the fact that any disease is the manifestation not only of genetic endowment but also of environmental factors particularly those affecting living conditions

As with other aspects of the relationships between living organisms it is not possible to comprehend the course of infectious processes without some knowledge of the past histories of the hosts and the parasites concerned there is no perspective without retrospective Microbial diseases have changed greatly in prevalence and in severity during historical times and the study of their evolution has provided much valuable knowledge concerning the role of hereditary factors in the resistance of man to the various kinds of infection The information so far gained strongly suggests that the hereditary levels of innate resistance and susceptibility are not necessarily racial characteristics but are rather an expression of the extent and the duration of the contacts that a particular group of people has had with a particular agent The history of disease among the Amerindians and the Polynesians is instructive in this regard

Smallpox apparently was introduced into the American Continent early during the Spanish Conquest, probably by a Negro in Cortez band The Indians proved to be highly susceptible to the disease which almost wiped out some of their settlements and there is reason to believe that this disaster contributed to their rapid defeat by the Spaniards The conquest of North America a century later provided further dramatic evidence of the susceptibility of the Amerindians to smallpox Repeated outbreaks decimated village after village and at times whole tribes Early in the 17th century, for example, the Massachusetts and the Narragansett Indians were reduced in a short time from 30 000 and 9 000 respectively to a few hundreds In the epidemic of 1837 the Mandan population fell from 1 600 to 31 the Assiniboins lost whole villages the Crows one third of their population while the total deaths among Plains tribes amounted to 10 000 in a few weeks Similar outbreaks occurred as late as 1870-71 among the Blackfeet

Other microbial diseases brought in by the European invaders contributed still further to the rapid decline of the Indian population during the 19th century There were more than 700 000 Indians in North America before the arrival of the Europeans but only 250 000 in 1850 Tuberculosis played a large part in this holocaust as illustrated by the epidemics which decimated the Plains Indians of Western Canada In the 1890 s the annual death rate from tuberculosis in the Qu Appelle Valley reservation of Saskatchewan reached the fantastic figure of 9 000 per 100 000 population More than half the Indian families were eliminated in the first three generations of the epidemic Moreover some 20 per cent of the deaths in the surviving families were caused by tuberculosis

Microbial diseases among the Polynesians during the past century present a picture similar to that seen among the Amerindians The South Pacific was explored during the second half of the 18th century Granted that the charm of the Pacific Islands and the amorous welcome of their women may have

warped the judgment of the European visitors there is no reason to doubt the validity of their unanimous opinion concerning the health and the vigor of the Polynesian people at that time The Europeans *saw in all the Islands robust and happy men and women obviously well adapted to their environment* including their local pathogens Yet disease became rampant among the islanders within a short time after these early explorations and the Polynesian population soon began to fall From approximately 300 000 at the time of Cook s first visit in 1778 the population of native Hawaiians had fallen to 37 000 in 1860 During the same period the population of the New Hebrides was reduced to one tenth of its original size

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they have been established for several generations Similarly it has been observed that after a while African trypanosomiasis loses the terrific virulence that it exhibits when first introduced into new districts of Equatorial Africa The disease lingers on but it carries off small numbers of victims instead of killing two thirds of the exposed population as it once did

Precise observations are available concerning the evolutionary changes in the clinical manifestations of tuberculosis among the Indians of North America During the first and the second generations of the tuberculosis epidemic in the Qu Appelle Valley reservation extensive glandular involvement was the rule in school age children Meningitis generalized miliary disease and bone and joint disease were extremely frequent—evidence of inability of the host to localize infection In 1921 at a time when the generalized epidemic was in the third generation the disease showed a greater tendency to localize in the lung and to exhibit a chronic course the mortality was falling and glandular involvement had dropped to 7 per cent among Indian school children This latter manifestation of high susceptibility to the disease has continued to decline steadily and is now seen in less than 1 per cent of children in the present (fourth) generation In other words while tuberculosis among the Amerindians exhibited at first a very acute course different in character from that observed in people who have had contact with the tubercle bacillus for several generations it is now undergoing a change which makes it resemble the more chronic type of disease commonly seen in the Western World under ordinary conditions

GENETIC FACTORS INVOLVED IN THE VIRULENCE OF MICROBIAL DISEASES

In order to account for the changes which have been observed repeatedly in the relation between man and agents of disease one may postulate that the latter undergo fluctuation in their infectivity or pathogenicity and that the resistance of men also varies from one generation to another First we shall discuss these possibilities then consider how changes in the environment can modify the manifestations of infectious processes

It is well known that viruses, bacteria and other parasitic agents can undergo in the laboratory mutations affecting most of their characteristics for example their immunologic specificity and their virulence While it is legitimate to assume that similar mutations also occur under field conditions so far there has not been any convincing evidence that this phenomenon plays an important role in affecting the course of human epidemics Recently however observations made with the virus of rabbit myxomatosis in Australia point to the possible importance of changes in virulence under natural epidemic conditions

The European rabbit was introduced in Australia in 1859 Finding no natural enemies in its new habitat the rabbit multiplied enormously (to several billions) and became an economic plague For this reason the virus of myxomatosis was introduced in Australia in 1950 in an attempt to control the rabbit population Myxomatosis occurs naturally in the wild rabbits of Brazil but merely in the form of a benign tumor producing infection with a very transient viremia The relationship between the Brazilian rabbit and the myxoma virus clearly denotes an ancient association resulting in ecologic equilibrium In contrast the virus recovered directly from Brazilian rabbits causes an acute almost uniformly fatal disease when inoculated into European rabbits

The initial outbreaks of myxomatosis in Australia were characterized by an enormously high case mortality rate—higher than 99 per cent Within a year however the case mortality had fallen to 90 per cent in areas where a second spontaneous outbreak had occurred This fall apparently was due in part to a decrease in the virulence of the virus In Australia the virus is transmitted from rabbit to rabbit almost entirely through mosquitoes which act mechanically as 'flying needles Because the highly virulent strain of virus kills rabbits within a very few days the chances for its transmission through the mosquito vector are rather limited However when a virus of lower virulence appeared spontaneously by mutation it produced in the rabbit a less rapidly fatal disease with skin lesions of longer duration Thus the less virulent mutant strain had a better chance of being transmitted through mosquito bite and

it progressively displaced in the field the original highly virulent strain A few years after its introduction in Australia the virus caused a mortality of 90 per cent in European laboratory rabbits instead of 99 per cent as it had originally

The evolution of rabbit myxomatosis in Australia has brought to light another important principle of epidemiology, by providing evidence of the fact that hereditary changes in the host can also be of importance in altering the course of epidemics As already mentioned the European type of rabbit introduced in Australia is immensely susceptible to myxomatosis a disease which did not exist in Europe until a few years ago As a result the mortality among infected animals proved to be almost total the first year that the virus was released successfully in Australia and Europe Recently however it has been found that rabbits trapped in areas of these countries where the infection has been prevalent for several years exhibit a degree of resistance to the most virulent forms of the virus much higher than that exhibited by rabbits before the beginning of the epizootic Furthermore it has been established that this increase in resistance has a genetic basis and therefore must result from the selection of mutant animals which had survived the initial infection

It is not easy to prove that genetic changes in the resistance of man to his pathogens also occur in the course of widespread human epidemics Nevertheless it seems legitimate to believe that since epidemics with a great killing power tend to eliminate a large percentage of persons having a high degree of susceptibility the likely outcome is the selective survival and multiplication of those endowed with a higher than average innate resistance The history of family groups strongly suggests that this mechanism operated in Europe and America during the great 19th century epidemics of tuberculosis as it certainly did when tuberculosis eliminated half the families among the Indians of the Qu Appelle Valley reservation

It can be shown that a slight loss of virulence by the parasite and some increase in the genetic resistance of the host provides the right setting for the various manifestations of acquired immunity to come into play in a large percentage of infected individuals Here again rabbit myxomatosis in Australia constitutes an enlightening model Protective antibodies have been found in the surviving animals trapped in infected areas Therefore it can be surmised that the maternal transmission of these antibodies helps the young to withstand infection during the first days of life and allows them to develop subsequently their own active immunity

The many interrelated aspects of this problem cannot be discussed here but it seems to be worth pointing out that the genetic and immunologic changes favoring resistance to infection which occur during a generalized epidemic are the almost inevitable outcome of prolonged contact between any parasite and any host For this reason it seems unlikely that any living species has ever been completely wiped out by infection however virulent the parasite The outcome of evolutionary forces is of necessity a *modus vivendi* according to which the parasite and the host reach some sort of equilibrium which permits the survival of both The concept of successful parasitism so ably developed by Theobald Smith and Swellengrebel a generation ago corresponds to this evolutionary equilibrium resulting from prolonged racial contact between host and parasite

THE CARRIER STATE AND THE PHENOMENON OF LATENT INFECTION

At the ultimate stage of evolutionary adaptation between parasite and host in a given community infection is extremely prevalent but rarely evolves into fatal disease—a situation referred to earlier in this chapter as peaceful coexistence

An extensive and rather confused terminology has grown out of the multiplicity of observations on the persistence of pathogens in the tissues of apparently normal individuals The expressions carrier state latency or dormancy of infection masking and unmasking of viruses etc have never been clearly differentiated one from the other More recently it has become the practice to designate as persisters the parasites which survive in tissues despite successful and prolonged chemotherapy The recognition that even unicellular organisms normally carry particles which are

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capable of multiplying intracellularly under special circumstances (for example, bacteriophages for bacteria, kappa factors for Paramecia) has contributed still further to the confusion by encouraging the introduction of new words. While discussion of nomenclature is outside the range of the present essay, it seems important to point out that the persistence of pathogens without manifestation of disease can occur in a wide spectrum of forms, differing both quantitatively and qualitatively. At one extreme are those situations in which the presence of the pathogen in the tissues can be demonstrated readily by standard technical procedures. The recovery of typhoid bacilli from the excreta of typhoid carriers or of the virus of herpes simplex from the tissues of the herpetic individual are examples in point. At *the other extreme are the cases in which re*covery of the pathogen is all but impossible as long as the infected individual is in a normal healthy state. This aspect of the problem usually is illustrated by examples taken from the filterable virus field but rickettsial and bacterial infections also provide striking examples of the phenomenon. Suffice it to mention the relation of Brill Zinsser disease to the unsuspected presence for several decades of *Rickettsia prowazeki* in the tissues of the victims. Or again one can point to pseudotuberculosis of rodents caused by corynebacteria which cannot be isolated from the animal in the normal state yet multiply explosively as a result of stress or of treatment with massive doses of cortisone.

It is certain that technical difficulties often account for the failure to isolate the pathogen. In the case of many viruses and also of certain bacteria the isolation technics are so inadequate that they yield positive results only when millions of active infective units are present in the sample tested. It is only in specialized cases that the technics permit detection of a single or a few infective units. The report that a pathogen is "masked" must always be judged therefore in terms of the sensitiveness of the procedure used to test for its presence.

There is good reason to believe on the other hand that peculiarities of the infected tissues often contribute to the difficulties encountered in revealing the presence of the pathogen. Such is the case for example when these tissues contain large amounts of substances which although not capable of destroying the pathogen can inhibit its multiplication. Thus neutralizing antibody transferred along with a virus is likely to prevent the latter from becoming established in a recipient animal. Tissue inhibitors other than antibodies can also cause difficulties, for instance, many tissue constituents can prevent the growth of tubercle bacilli even in highly favorable culture media. Elimination of neutralizing antibodies and of other inhibitory agents would be a prerequisite for the successful demonstration of the presence of small numbers of infective units in the tissues but few if any are the cases where this can be done adequately.

Even more subtle mechanisms may be involved in the failure to recover pathogens from infected tissues. Some of the recent advances in knowledge concerning the structure of viruses and bacteria are of suggestive interest in this regard as illustrated by the following examples. The tobacco mosaic virus has *been shown to be made up of a nucleic acid* unit enclosed in a protein tubular structure. The nucleic acid component is solely responsible for virus reproduction, whereas the protein acts as a protective coating and thereby increases the resistance of the virus. A situation having some analogy (at least formal) to that found with regard to viral structure has been recognized in bacteria. Under usual conditions the bacterial cell is surrounded by a rather rigid wall which protects its plasma membrane. However, it is possible by the use of certain technics (for example treatment with penicillin or lysozyme) to prepare bacterial protoplasts devoid of cell wall. These protoplasts are endowed with complex synthetic powers and can reproduce the whole cell under the proper conditions but they are highly susceptible to osmotic disturbances and to other deleterious effects. Because of this susceptibility the protoplasts are rapidly destroyed in the usual laboratory media and in consequence can multiply only under very special conditions. On the basis of all these facts it may be wondered whether certain viruses and bacteria do not persist in vivo in the form respectively of naked nucleic acid structures or of protoplasts sheltered in the well balanced and constant milieu provided by the internal environment of tissue cells. If this were the case it probably would follow that such delicate infective structures could be readily inactivated once they were removed from their protective environment and as a result would usually escape detection by the usual laboratory procedures.

It must be emphasized that the points of view outlined above have not yet been documented by factual information. They are presented merely to illustrate that many different

mechanisms could account for the well established fact that pathogens often persist in the tissues even when it is impossible to demonstrate their presence by ordinary technics As no precise knowledge of the mechanisms involved is available at the present time for most of human infections it is not surprising that the terminology devised to describe these pheromena has remained confused Until agreement based on understanding has been reached it is hardly possible to set precise limitations to the meaning of the words in common usage In the present chapter we shall employ interchangeably and in a somewhat loose manner the expressions carrier state and dormant or latent infections without regard to any specific mechanisms merely to denote situations in which a pathogen persists in the tissue in one form or another without manifesting its presence by obvious signs or symptoms of disease

The fact that pathogens can exist and persist in the tissues without manifesting their presence by pathologic disorders has of course long been known As early as the 1840 s investigations of the potato blight in Ireland revealed that most potato plants normally carried the fungus *Phytophtora infestans* and that the parasite caused the blight only under unusual weather and growth conditions Pasteur observed that guinea pigs can harbor the bacilli of fowl cholera for many months in innocuous abscesses which do not seriously affect the health of these animals He clearly realized and emphasized the theoretical importance of this fact for epidemiology Koch proved that healthy men can excrete cholera vibrios in their stools and he thus provided the first practical illustration of the fact that healthy carriers can play a role in the transmission of the disease Clearly then the fact that infection can exist without disease was understood early long before the designation carrier state was coined for the concept and before Typhoid Mary became its personification But it is only during the past two decades that evidence has been obtained of the generality of the phenomenon namely of the fact that the pathogenic agents characteristic of a community do commonly become established in the tissues of a very large percentage of normal persons and yet cause clinical disease only in a very small percentage of them

Much of the progress in this field has grown out of the study of latent viral and rickettsial infections It has been established that even under the most careful and sanitary breeding conditions all laboratory animals carry a large variety of microbial agents potentially pathogenic for them as a part of their normal intestinal and respiratory flora and that the latent infections can be evoked into activity as the expression goes by all sorts of nonspecific stresses Most colonies of albino mice for example are infected with the pneumonia virus of mice (PVM) which is potentially capable of causing a fatal pulmonary disease in them but usually remains dormant Many flocks of birds of parakeets in particular are normally infected with the psittacosis virus but they do not suffer from the infection until the virus is caused to multiply by the stresses occasioned by poor husbandry The recognition that Brill s disease is the manifestation of reawakening of an old dormant infection with the epidemic strain of typhus provided the first well documented evidence that latent rickettsial infections can occur in man The recent isolation of several types of adenoviruses—as well as of many viruses in search of a disease —in the tonsils and the adenoids and other tissues of healthy persons makes it clear that latent virus infections are as common in men as they are in experimental animals

Latent infections with bacteria fungi and higher parasites are at least as common as are those with viruses and rickettsia To quote only a few examples All surveys indicate that some 30 to 50 per cent of normal persons harbor coagulase positive staphylococci in their nasopharynx until a few years ago practically all human beings in the Western World did become infected with tubercle bacilli and this situation still exists today throughout Asia in the United States *Amoeba histolytica* can be isolated from the stools of a surprisingly large number of persons who have never suffered from amebiasis some 30 per cent of the population give a positive skin test for toxoplasmosis and encysted trichina are often found in muscles in the absence of any clinical sign of trichinosis The list of dormant infections with all classes of pathogens continues to increase as the search for them is expanded Furthermore there is now overwhelming evidence that many micro-organisms—long

thought to be of no pathogenic importance—which are part of the autochthonous flora of normal tissues often become involved in disease processes when the general resistance of the body is decreased by any cause whatever

Thus it is becoming apparent that the carrier state is not a rare immunologic freak In reality infection without disease is the rule rather than the exception But latent infection can become activated under certain conditions For this reason a large and most important aspect of the epidemiology of disease (as contrasted with the epidemiology of infection) has to do with the factors that upset the equilibrium between host and parasite and thereby convert dormant infection into overt disease

ACTIVATION OF DORMANT INFECTIONS BY ENVIRONMENTAL FACTORS

It is clear of course that maintenance of health in the face of persisting infection means that the mechanisms of acquired immunity (humoral or cellular) are able to inhibit the multiplication of parasites but are not capable of eradicating them from the tissues On the other hand it must be emphasized that acquired immunity does not necessarily mean *freedom from disease Many types of non* specific disturbances are known to allow extensive multiplication of parasites even in individuals endowed with a high degree of acquired specific immunity to them For example persons who carry the virus of herpes simplex have a high level of neutralizing antibodies for this virus in their serum Nevertheless transient episodes of viral multiplication can take place in infected persons under the influence of nonspecific stimuli with the result that herpes blisters develop in the presence of persisting humoral immunity to the virus

On the basis of clinical experience as well as of lay observations it is usually considered self evident that susceptibility to all sorts of infections is much increased by the various stresses and strains of everyday life Patients as well as their physicians tend to incriminate bad weather, lack of sleep, poor constitution and emotional disturbances even in the causation of diseases known to be the result of activities of viruses or bacteria In reality few are the cases in which a convincing correlation has been established between the physiologic state of the host and his susceptibility to infection Diabetes constitutes probably the best documented example of this type of relation, *since there is no doubt that the poorly* controlled diabetic patient is easy prey to staphylococci, tubercle bacilli and other bacteria It is also true, on the other hand, that his resistance to infection becomes almost normal once the diabetes is controlled by adequate insulin therapy In this case, therefore, the response of the host to infection is under the influence of events, probably biochemical in nature which can be altered reversibly by the physiologic control mediated through insulin

It is of theoretical and great practical importance that certain therapeutic procedures also can increase susceptibility to infection As shown in another chapter the very use of antimicrobial drugs not uncommonly favors *the multiplication in vivo of micro organisms* which are not susceptible to these drugs Extensive surgery and any form of trauma especially if it results in a state of shock also can render the defense mechanisms of the body partially ineffective at least for a while This is also true of the use of radiation for therapeutic purposes—a fact which probably con*tributes to the prevalence of staphylococcal* and other infections among patients treated for malignant diseases Many of these phenomena have been reproduced and studied in the laboratory during recent years Thus the susceptibility of experimental animals to certain types of infection—most interestingly to the microbial agents that they normally carry in their tissues—can be increased at will by administration of antimicrobial drugs by production of traumatic shock or by extensive body irradiation

History provides many examples of the fact that social misery and its usual consequence physiologic misery is commonly associated with increase in the prevalence and the severity of microbial diseases War famine and pestilence have been known to ride together throughout history In many cases of course the infectious diseases associated with war are directly the result of increased contact with particular types of microbial agents Armies in the field have been plagued by typhoid and dysentery whenever the breakdown of sani

tary practices has permitted massive infection with salmonella and shigella Napoleon s troops contracted typhus when they became saturated with lice and rickettsia during their march through Eastern Europe in the 1812 Russian Campaign Similarly the Western allies suffered much from scrub typhus and malaria in the Asiatic theater during World War II even though the adult native population suffered little from these diseases to which they had been exposed throughout life

Of equal importance and greater interest perhaps is the fact that wars and other social upheavals can increase the prevalence of microbial disease through other mechanisms namely by creating conditions which decrease the general resistance of the body

The tuberculosis epidemic which prevailed throughout the Western World during the 19th century owed at least part of its severity to the long working hours the poor nutrition and the low living standards prevailing among the labor classes involved in the Industrial Revolution As living conditions improved tuberculosis mortality began to decrease spontaneously The change was already noticeable at the end of the century long before any specific measure of prophylaxis or therapy had been introduced Tuberculosis mortality again exhibited a sharp and sudden rise in most of Europe during World Wars I and II and once more it resumed its downward course as soon as the hostilities were over During the 1920 s inflation in Germany provided a spectacular illustration of the bearing of social factors on resistance to disease Following an abrupt fall at the end of the war the tuberculosis mortality increased sharply during the years of inflation and fell again as soon as stabilization of the currency allowed a return to more normal living conditions The rapid and reversible manner in which the social body responds *to changes in living conditions by changes in* tuberculosis mortality rates cannot be explained in terms of infection rates The more likely explanation is that large numbers of persons in our communities are in a state of unsteady equilibrium with tubercle bacilli as well as with many other pathogens and that this equilibrium is upset whenever disturbances impair the general state of resistance of the body

This relationship has been brought out in a recent analysis of the disease problems in German concentration camps during World War II Among infectious diseases it was not the exotic unusual epidemics like typhus cholera or even bacillary dysentery which proved to be the most troublesome in the camps but rather ordinary skin ailments colds bronchopneumonias staphylococcus infections pulmonary tuberculosis—in other words the type of diseases minor or severe caused by micro-organisms *endemic* in the normal European communities Here again increase in contact infections could hardly account for the aggravation of these endemic diseases Far more important certainly was the loss of natural resistance caused by malnutrition and other forms of physiologic misery It was remarkable indeed that most of the internees overcame their microbial maladies shortly after their return to a normal environment and often without the help of specific therapy Even in the case of tuberculosis rapid recovery was the rule though no antimicrobial agent was then available for its treatment

While there is general agreement concerning the fact that many nonspecific stresses increase the vulnerability of the host there is but little if any understanding of the mechanisms through which these effects are exerted Indeed many of the relationships that are believed to be so obvious as to need no demonstration have proved to be very difficult to reproduce in the laboratory for example the effect of nutritional deficiencies or of emotional upsets on susceptibility to infection Even experiments in human volunteers have failed to provide experimental evidence that exposure to cold and other forms of bad weather does in any way facilitate infection with the cold virus For this reason it has not yet been feasible to identify in physiologic *and biochemical terms the mechanisms* through which non pecific stresses alter susceptibility to microbial disease

Students of infection have long been intrigued by the possibility that hormonal influences play an important role in controlling the body response to micro-organisms a su picion highlighted recently by the finding that the activity of the adrenal cortex is related in various ways to the phenomena collectively grouped under the word stress The most

concrete achievement in this field has been the recognition in the clinic and demonstration in the laboratory, that treatment with large doses of cortisone can bring about indirectly an increase in the multiplication of pathogenic agents and indeed often evokes latent infections into activity It has been shown furthermore that ACTH can also enhance infection in certain experimental models and that its enhancing effect can be neutralized by the proper dose of somatotrophic hormone Hypothyroidism seems to be associated with susceptibility to certain infections and contrariwise there is evidence that injection of thyroid hormone into rabbits markedly increases their resistance to experimental tuberculosis The influence of diabetes and insulin on susceptibility to bacterial infection in man has been mentioned already Thus it is certain that the course of infectious processes can be altered profoundly by a variety of hormonal influences, but the intimate mechanisms involved are far from clear

The fact that cortisone interferes with the production of antibodies under certain conditions seems at first sight to provide the clue to the problem However for reasons not to be presented here this immunologic inhibition cannot account entirely if at all for the infection enhancing effect of the hormone In addition to inhibiting antibody production cortisone influences many other physiologic processes some of which may affect directly or indirectly the response of the body to infection Interference with the inflammatory response and with the activity of the reticulo endothelial system disturbance of intermediate metabolism activation of proteolytic enzymes are some of the effects of cortisone recognized so far that are worth considering in this regard Instead of discussing them at this time, it seems to be more convenient to analyze their influence in the light of a more general review (to be presented in the following section) of the natural mechanisms through which the body deals with microbial agents

NORMAL FACTORS OF RESISTANCE

Once pathogens have become established in the body there come into play mechanisms that tend to limit their spread beyond the site of the initial lesion One of the essential components of this walling off process is the deposition of fibrin of the sulfated polysaccharides of reticulum and finally of collagen The very nature of this reaction involving as it does the synthesis of large amounts of special polysaccharides and proteins points to the possibility that it can be interfered with by many metabolic disturbances For example nutritional deficiency in vitamin C or in sulfur containing dietary constituents is known to retard the production of reticulum similarly quantitative or qualitative inadequacies in intake of protein or amino acids can handicap the production of fibrin and collagen It has been shown in fact that the rate of healing of wounds is influenced markedly by these dietary factors

In addition to the metabolic disturbances that interfere with the biochemical syntheses involved in producing the materials required for walling off the lesion there are other pathologic processes which tend to break down its protective structures for example by destroying fibrin and collagen Thus it has been shown by electron microscopy that collagen fibers undergo softening and do disintegrate at the site of an Arthus reaction Of further interest in this regard is the fact that many types of stimuli—as unrelated in origin as antigen antibody reactions emotional upsets or injection of large doses of cortisone—can activate the potential proteolytic activity of body fluids and tissues It is conceivable that these various stimuli exert an unfavorable influence on the evolution of disease indirectly through the proteolytic breakdown of the barriers deposited at the site of lesions

There is evidence also that physiologic disturbances can interfere with natural resistance by inhibiting the forces of the body which exert a protective effect more directly through a deleterious effect on infectious agents Blood and other body fluids and tissues are known to contain a variety of substances which are injurious to pathogens in vitro While there is as yet no convincing evidence that these substances exhibit any significant anti infectious activity in vivo it is worth noting that the blood level of at least one of them properdin has been shown recently to undergo profound variations depending upon the state of the individual

There is no doubt of course that phago

cytosis by polymorphonuclear cells circulating and fixed monocytes as well as by other cells of the reticuloendothelial system play an essential role in defense against infection. Unfortunately little is known of the mechanism through which phagocytes destroy the pathogens that they engulf or restrain their multiplication. Polymorphonuclear leukocytes contain two basic proteins—lysozyme and phagocytin—which together have a very wide range of bactericidal activity and probably contribute to the well known bactericidal power of these cells. Whether the synthesis and the activity of lysozyme and phagocytin undergo variation with the physiologic state of the individual is still a moot point.

No substance of activity comparable with that of lysozyme or phagocytin has yet been separated from phagocytic cells other than polymorphonuclear leukocytes but there is reason to believe that the very process of phagocytosis and the complex of phenomena associated with the inflammatory response create metabolic situations which are inimical to many pathogenic agents. It is known for example that phagocytosis brings about a burst of acidity which brings to a very low level the intracellular pH of the phagocyte in large part probably a result of the production of lactic acid through glycolysis. Furthermore it has been shown by several independent technics that inflammatory areas also contain a high concentration of lactic acid and that their reaction may be very acidic at least during the early phases of inflammation. There is evidence on the other hand that lactic acid can inhibit most microbial agents including viruses and can even cause their destruction especially at the low pH the low oxygen tension and the high CO_2 tension prevailing locally at the inflammatory site.

In summary it appears that one of the results of phagocytosis and of the inflammatory response is to create locally an environment that is inimical to the multiplication and the survival of pathogenic agents through the local accumulation of phagocytic cells and of anti infectious substances as well as through the production of an abnormal metabolic environment at the site of the lesion. It would follow in consequence that any factor capable of modifying either quantitatively or qualitatively the various aspects of inflammation is likely to express itself in a change of susceptibility to infection. Cortisone is one but probably only one of the several hormones which play a role in this regard by interfering both with the influx of inflammatory cells and by altering their metabolism. The metabolic state of the infected individual is also certainly of importance since it affects the biochemical activities of phagocytic and other inflammatory cells. For example it is possible that the metabolic products of these cells during the ketosis of *uncontrolled* diabetes or during the acute phase of starvation differ from those characteristics of the normal healthy state. It is also likely that the vascular disturbances associated with any emotional upset influence the gaseous and metabolic exchanges at the site of the lesion and thus secondarily affect the fate of the microorganisms that it harbors through an alteration in the local biochemical environment.

DETERMINANTS OF DISEASE

While there is as yet no precise information concerning the mechanisms through which the body manages to ward off infection under normal circumstances there is convincing evidence that these mechanisms can be rendered ineffective by many of the stresses known to be associated with increased susceptibility to infectious disease. For example alloxan diabetes injection of cortisone or of bacterial endotoxins exposure to radiation have been shown in experimental animals to decrease the clearing power of the tissues for various types of micro organisms. This decrease in clearance is the expression of either one or both of two different effects—interference with the ability of phagocytic cells to engulf the pathogens or interference with their ability to destroy the pathogens after phagocytosis. It is also easy to demonstrate experimentally that minor disturbances can act in a highly localized and transient manner to alter the course of an infectious process. Thus intradermal injection of doses of epinephrine so small as to cause a blanching of only a few minutes duration is sufficient to render the conditions at that *particular site* favorable for the multiplication of small doses of bacteria which otherwise would fail to proliferate in an unprepared site.

Thus epidemiologic evidence and experi

mental facts make it clear that the ecology of microbial disease is under the influence of factors both general and local that are independent of those which control the frequency of contact with infectious agents The relative importance of the factors which determine the chance that infection will take place, and *of those responsible for converting latent infection* into disease is conditioned naturally by the characteristics of each particular microbial agent but also varies from one population and one community to another When a group of people (whatever their race) first comes into contact with a pathogen the chances are great that the general mechanisms of resistance will be of little avail and that infection will become manifest in the form of severe disease in a very large percentage of cases This type of situation developed when smallpox measles tuberculosis etc were first *introduced among the Amerindians and other* primitive people It was produced experimentally by introducing the myxoma virus among the rabbits in Australia and Europe it probably would happen again if yellow fever plague or any types of infections with which western man has not had wide contact recently were introduced on a large scale in the communities of the Western World today Conceivably it could occur if new microbial agents were introduced among men animals or plants for the purpose of biologic warfare Whether mutants of common pathogens can behave as new agents of disease against which the general mechanisms of resistance would be of little avail is a theoretical possibility that has been suggested to account for the virulence of widespread epidemics like the 1918 influenza but for which no evidence is yet available

At the other extreme are the epidemiologic situations in which a particular pathogen is ubiquitous in a given community and becomes established in the form of a latent infection in most normal individuals Since infection is not one of the variables in this type of epidemiologic system the factors that up set *the ecologic equilibrium between host and* parasite then become the effective determinants of disease Needless to say there are many intermediate situations between these two extremes and the evolution of microbial diseases leaves no doubt furthermore that changes can occur rapidly in the parasite, in the host and in the type of relationship that exists between them How rapidly the change can occur has been shown with choriomeningitis virus in mice This virus produced a severe disease with fatal outcome when first introduced among albino mice but eventually *it became established* in several mouse colonies Within a few years after its introduction the virus was found to be present in all animals of these colonies producing in them a latent infection The infection was contracted in utero but did not manifest itself by any detectable sign of disease in adult animals even though their organs continued to harbor large amounts of active virus throughout life In this case therefore, a few years had sufficed to change the type of relationship between mice and choriomeningitis virus from that of a virulent epidemic to a *state of silent commensalism or symbiosis The* virus had become an unobtrusive guest whose presence was barely noticed by the host

It is clear in conclusion that the type of relationship existing at any given time between hosts and their parasites is the outcome of many different factors including past racial experience evolutionary adaptation through genetic changes and immunologic processes transient disturbances in the internal and the external environments Because of the dynamic character of this relationship the methods used in the control of microbial diseases both prophylactically and therapeutically must differ from place to place and vary from time to time The methods of sanitation and vaccination that were designed to cope with the great epidemics of the past may not prove to be effective in the control of the disease states caused by microbial agents which are ubiquitous in our communities in the form of dormant infections So far the main goal of medical microbiology has been to treat established disease and ideally to prevent infection from taking place through technics designed for aggressive warfare against microbial agents It might be worth *considering* now whether useful practices of disease control can be derived from the fact that peaceful coexistence with pathogens often occurs in nature To this end it will be necessary to separate conceptually and factually the determinants of infection from the de

terminants *of disease* and to try to bring under control the processes which are responsible for converting infection into overt disease

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3

Morphology, Physiology and Genetics of Bacteria

BACTERIAL MORPHOLOGY *

Shape and Dimensions

Bacteria are a morphologically varied group of unicellular organisms of microscopic size Depending on the species they may vary in size from a fraction of 1 micron to a few dozen microns but most frequently they are of the order of one to several microns Their shape can approximate that of a sphere (coccus), of a straight rod (bacillus) or of a curved rod (vibrio spirillum) These morphologic characteristics examples of which are shown in

* Detailed bibliographies concerning this topic can be found in Dubos (1945) Knaysi (1951) and Spooner and Stocker (1956)

Fig 1 Some morphological types of bacteria All but G are from culture in artificial media

(A) Large coccus from human sputum Impression smear gelatin culture stained with fuchsin

(B) Pneumococci One day old culture stained by Gram technic

(C) Large sarcina (*Sarcina agilis*) living cells in hanging drop faintly stained with methylene blue

(D) Diphtheria organisms (*Corynebacterium diphtheriae*) Involution forms stained with methyl violet

(E) Anthrax bacilli gelatin culture stained with eosin methylene blue

(F) Botulinus organisms (*Clostridium botulinum*) Glucose gelatin culture showing endospores

(G) Gas gangrene organisms (*Clostridium sp*) in guinea pig tissues stained with methylene blue showing terminal spores

(H) Tetanus bacteria (*Clostridium tetani*) showing terminal spores (drumsticks) Agar culture stained with fuchsin

(J) Anthrax bacilli Agar culture stained with fuchsin and showing central spores

(K) Dysentery organisms (*Shigella sp*) Agar culture stained with fuchsin

(L) Influenza bacteria (*Hemophilus influenzae*) Culture stained with fuchsin

(M) Blue pus organisms (*Pseudomonas aeruginosa*) Agar culture stained with fuchsin

(N) Glanders organisms (*Malleomyces mallei*) Potato culture stained with fuchsin

(P) Plague bacteria (*Pasteurella pestis*) Culture on 3 per cent salt agar showing involution forms stained with methylene blue

(Q) Plague bacteria showing capsules Culture 6 to 7 days old with ferrotannate as mordant according to Loeffler stained with fuchsin Unless otherwise stated all reproductions are 1 000 × Details of the photographic technic are given in the source quoted below The small numbers (in black) seen in the microphotographs are those appearing in Zettnow s Atlas (Zettnow E Atlas Photographischer Tafeln nach Originalaufnahmen *in* W Kolle and A Wassermann Handbuch der pathogenen Mikroorganismen Jena Fischer 1902)

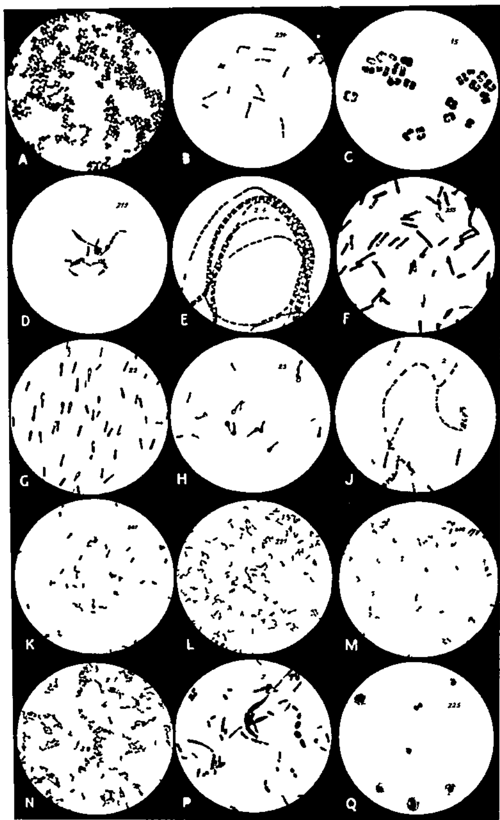


FIGURE 1 *(Caption on facing page)*

Figures 1 and 2 are relatively constant within taxonomic subdivisions and hence are of considerable significance in the classification of bacteria (see p 79) However even within as low a taxonomic subdivision as the species, there can be considerable variability in shape and size Such variability can be the result of environmental changes, like those occurring in aging cultures or of genetic alterations that may arise even under constant environmental conditions Apart from differences in size and shape bacteria may occur in a variety of spatial arrangements for example bacterial cells may occur singly (Fig 1 M), in chains of two or more (Figs 1 B and 2 A), in regular packets (Fig 1 C) or in irregular aggregates (Fig 1 A) In addition some bacteria particularly members of the order *Actinomycetales* form multiply branched filamentous structures known as mycelia

Of the major groups of bacteria (which include eubacteria or true bacteria chlamydobacteria, myxobacteria spirochetes pleuropneumonialike organisms and rickettsiae), the eubacteria represent the most extensively analyzed group from the standpoint of cellular anatomy Since their anatomic features are in many respects representative of bacteria as a whole we shall use them as a model in the discussion to follow

A Representative Eubacterial Cell

In spite of differences in size, shape and cellular arrangement, there are sufficient similarities in the general structural features of bacterial cells to permit a diagrammatic representation of a 'typical' eubacterium as shown in Figure 3 In this idealized sketch three major components are discernible (1) a *cell envelope*, comprising capsule cell wall and cell membrane (2) *cytoplasm* and its inclusions and (3) a *nucleus* In addition, as indicated in Figure 3 some bacteria possess one or more flagella which, though extracellular in gross appearance originate in the cytoplasm and extend through the cell envelope Each of the major components plays a vital role in the life of a bacterium therefore their structure and chemical properties will be reviewed individually in the following section

Cell Envelope Component parts of the cell envelope can be differentiated by direct microscopic observation by analyses of their chemical characteristics and by the selective removal of successive component layers

Capsule The outermost structure of many but not all bacteria is a distinct layer known as capsule (Fig 4), which contains substances of high molecular weight The nature of capsular substances may vary from one major

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Fig 2 Bacteria as they occur in infected tissues

(A) Streptococci in meningitis (spinal fluid) stained with fuchsin

(B) Pneumococci in exudate from human lung stained with aniline water and fuchsin

(C) Ozena organisms (*Klebsiella ozenae*) in human nasal secretion very old smear stained with methylene azure

(D) Diphtheria organisms in mucus from the trachea stained with fuchsin

(E) Anthrax bacilli in mouse spleen stained with fuchsin

(F) Leprosy organisms (*Mycobacterium leprae*) Smear from nasal mucus stained by Ziehl Niehlsen Note that the bacilli appear to be associated with the nucleus of the leukocytes

(G) Gonococci in pus stained with methylene blue

(H) Meningococci Section through the inner meninges stained with borax methylene blue (500 ×)

(J) Influenza organisms in sputum stained with fuchsin

(K) Mouse typhoid organisms (*Salmonella typhimurium*) in mouse spleen stained with fuchsin

(L) Coliform organisms in urine stained with fuchsin

(M) Cholera stool showing predominantly cholera vibrios a few large rods and cocci are also seen among the comma bacilli

(N) Fowl cholera organisms in chicken blood stained with formol gentian violet

(P) Plague bacteria in rat spleen stained with methylene blu

(Q) Relapsing fever spirochetes in human blood stained with fuchsin

Unless otherwise stated all reproductions are 1 000 × Details of the photographic technic are given in the source quoted below The small numbers (in black) seen in the microphotographs are those appearing in Zettnow s Atlas (Zettnow E Atlas Photographischer Tafeln nach Originalaufnahmen *in* W Kolle and A Wassermann Handbuch der pathogenen Mikroorganismen Jena Fischer 1902)

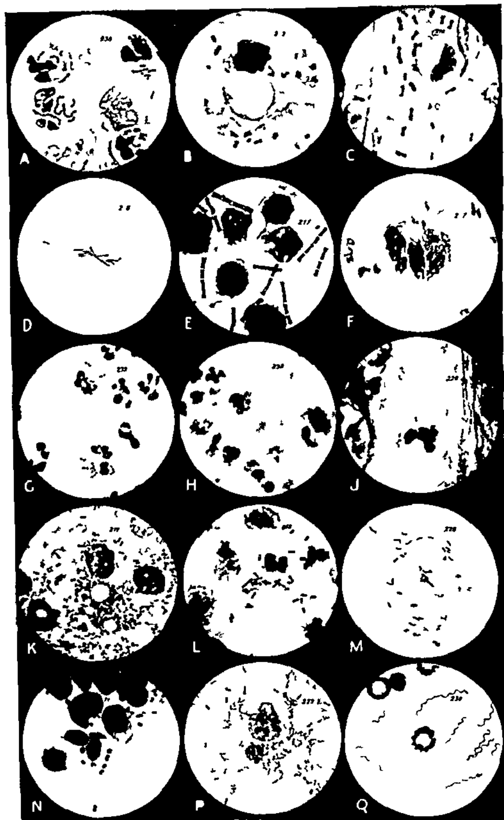


FIGURE 2 *(Caption on facing page)*

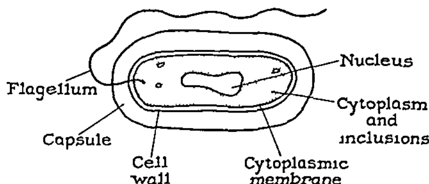


FIG 3 Diagrammatic representation of a eubacterial cell

taxonomic group to another and even from strain to strain but the most frequently encountered capsular materials are polysaccharides and polypeptides Thus virulent pneumococci possess polysaccharide capsules which differ in chemical structure from type to type, many streptococci form capsules containing hyaluronic acid virulent anthrax bacilli are known to produce under the proper environmental conditions a capsular material consisting of a polypeptide of D glutamic acid and plague bacteria may form capsules containing protein *and* polysaccharides As noted differences in the specific structure of a given capsular material may occur among strains of a single species such differences can be recognized by the specific antibody production following the injection of a given capsular material into suitable animals In many cases the detection of such immunologic specificity (type specificity) preceded the demonstration of differences in chemical structure of the macromolecules involved (Dubos 1945) Immunologic procedures have also revealed that regional differentiation in the composition of individual capsules may occur for example the capsules of certain strains of *Bacillus megaterium* consist of glutamyl polypeptide interspersed within a framework of polysaccharide (Tomcsik, 1956)

Capsules are not essential for the survival and the growth of bacteria (except in some specific environments to be discussed below) Capsular material can be removed, for instance, by treatment with suitable enzymes and the thus decapsulated bacteria will grow as well (in vitro) as do their encapsulated counterparts Similarly growth rate of spontaneously arising unencapsulated mutants may be similar to and sometimes even faster than those of their encapsulated parent cells Under certain environmental conditions however encapsulation can be of great significance for the survival of a bacterium Thus a substantial degree of protection against certain host defenses particularly against phagocytosis is afforded to many pathogenic bacteria by their encapsulation In this way, encapsulation by enhancing the in vivo survival of bacteria, can contribute to their pathogenicity (see also p 74)



FIG 4 Encapsulated *Bacillus megaterium* × 2 000 Bruce White stain (Dr H J Welshimer Medical College of Virginia)

CELL WALL Both in the absence and the presence of morphologically distinct capsules bacterial cells possess walls (Fig 5) of complex composition Carbohydrates polypeptides and lipids have been recognized as major components and it has been observed that the specific nature of each of these components and their relative proportions can differ among and within species In general walls of gram negative (see p 38) bacteria appear to be more complex than those of gram positive forms Gram negative walls contain sugar hexosamine lipid and protein components In the latter as many as 17 different amino acids

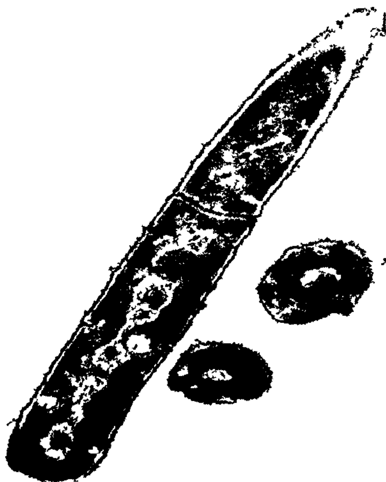


FIG 5 Electronmicrograph of (1 longitudinal and 2 transverse) ultrathin sections of *Bacillus cereus* × 60 000 Note relatively transparent nuclear region surrounded by more opaque cytoplasm also note complete as well as incomplete transverse cell walls (Chapman G B and Hiller J 1953 J Bact 66 362 373)

including aromatic and sulfur containing ones as well as proline and arginine have been detected In addition the amino acid diaminopimelic acid (DAP) not generally found in proteins has been discovered in the walls of gram negative bacteria (see also p 53) In contrast gram positive walls contain a more limited range of amino acids particularly conspicuous (in the cases so far examined) is the absence of aromatic and sulfur containing amino acid and of proline and arginine DAP is present in most but not all gram positive bacteria

Information such as cited above regarding the composition of cell walls has become available through chemical and immunologic analyses which have been applied either to whole cells or to isolated cell walls For the purpose of studying isolated cell walls various preparative procedures have become available which depend upon either mechanical or chemical disruption of bacteria followed by differential centrifugation

Cell walls of some bacteria are susceptible to more or less complete digestion by certain enzymes the best known is lysozyme which is capable of digesting the walls of certain gram positive organisms Following wall destruction lysis of the naked cells generally ensues unless they are suspended in a suitable hypertonic menstruum which prevents their rupture Under such protective conditions spherical wall deficient or wall free forms (protoplasts) capable of continued metabolism can be obtained (Weibull 1956) Similar protoplast formation also has been

achieved through penicillin treatment of certain gram negative forms in a medium made hypertonic by the incorporation of 20 per cent sucrose, in this instance the deficiency in wall material appears to be referable to the ability of penicillin to interfere with cell wall synthesis (Lederberg 1956a) From the lytic effects that occur as a result of significant reduction of wall material in the absence of a suitable hypertonic environment, the "corseting" function of the wall is clearly apparent In addition the wall serves as a barrier to the transport of molecules larger than the pore size of the wall which has been estimated to be of the order of 1 mμ in diameter

Since walls are surface structures they exert a significant influence upon various interactions between bacteria and their environment Among the characteristics of bacteria influenced by the chemical nature of the wall are antigenic and immunogenic properties as well as sensitivity or resistance to bacterial viruses to phagocytosis to bactericidal factors of the blood and tissues of animal hosts and to other environmental agents for which the wall is the primary site of attack

Cytoplasmic Membrane The innermost part of the cell envelope is a membranous structure which separates the cytoplasm i e the cell's principal site of metabolic activity, from the cell wall In the few cases so far examined this innermost structure often termed "cytoplasmic membrane has been found to be rich in lipoprotein One of the membrane s major functions is the control of the exchange of substances between the cell and its surrounding medium whereas the cell wall is generally considered capable of preventing only relatively large molecules from penetrating into the cell the cytoplasmic membrane appears to constitute an important osmotic barrier to small molecules Another important role of the membrane is suggested by its apparent association with certain enzymatic activities For example in some organisms the cytochrome system which participates in electron transport (see p 43) has been reported to be intimately associated with the membrane This conclusion rests upon the observation that following destruction of protoplasts by osmotic shock e g by immersion in water the residual baglike structure

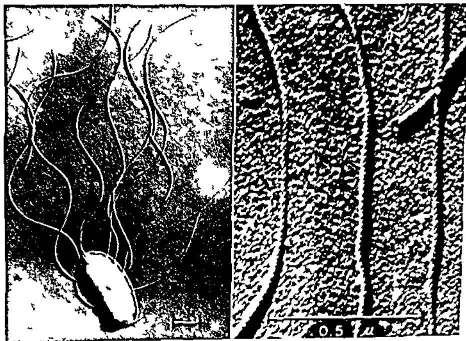


Fig 6 Palladium shadowed electronmicrographs of *Brucella bronchiseptica* with flagella (left at a magnification of 13 000) the periodic structure of the flagella is visible on the right at a magnification of 77 000 (Labaw L W and Mosley V M 1955 Biochim et Biophys Acta *17, 322 324*)

("ghost") which appears to contain the membrane also contains the cytochrome and other enzymatic systems (Weibull 1956)

Flagella Many bacteria possess threadlike structures flagella (Figs 3 and 6) which extend to the exterior through the cell envelope but have their origin in the cytoplasm The cytoplasmic origin of these organs of locomotion is indicated for example by the observation that following removal of the wall by lysozyme treatment flagella will remain attached to the resulting protoplast The number of flagella and their position on the cell body differ among species and provide taxonomic criteria However removal of flagella which can be accomplished by mechanical shaking or other means does not necessarily destroy viability Flagella are of protein nature and as shown by electron microscopy have a helical structure (Fig 6) The relatively simple structure of bacterial flagella is in graphic contrast with the more complex typically 11 stranded make up of the flagella or the cilia of other micro organisms plants and animals The protein of bacterial flagella differs from the cell wall protein and this difference is reflected in the antigenic specificity of such proteins For example so-called H antigens of *Salmonella* (see p 75) are associated with the flagella and can differ among strains a difference of importance in diagnostic procedures Antibodies against these H antigens will not react with nonflagellated forms but will react with corresponding flagellated bacteria In contrast so called O antigens are associated with the wall material and antibodies against them react with the corresponding bacterial type regardless of the presence or the absence of flagella

Cytoplasm and Its Inclusions The cytoplasm the major site of metabolic activities (see p 42) consists of an exceedingly complex mixture of low and high molecular components dissolved or suspended in an aqueous menstruum Water usually represents about 75 to 80 per cent of the cytoplasm Microscopically a wide variety of so-called cytoplasmic inclusions are discernible Among them are polysaccharide granules lipid globules and granules rich in polymetaphosphate (frequently described as *metachromatic granules* or *volutin*) Some of these cytoplasmic inclusions may be regarded as reserve materials therefore their nature and frequency

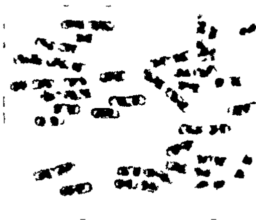


Fig 7 Nuclei in *Bacillus cereus* cells Fixed with osmium vapor and stained by acid Giemsa × 3 000 (C Robinow from Braun W Bacterial Genetics Philadelphia Saunders)

may fluctuate with the age physiologic condition and environment of the cell In addition bacteria possess a functional if not structural equivalent of mitochondria found in cells of higher forms (Mudd et al 1956) Apart from classical cytologic technics chemical and physical fractionation methods applied to disrupted cells have been useful in revealing the heterogeneity of bacterial cytoplasm

The Nucleus A distinct morphologic structure rich in deoxyribonucleic acid (DNA) the nucleus has been demonstrated to be embedded within the cytoplasm of bacteria (Fig 7) As in the case of higher organisms nuclei can be detected by various means particularly by staining reactions which depend upon the presence of DNA Thus the Feulgen reaction which involves the formation of a purple color from the interaction between reduced fuchsin and the aldehyde group liberated from DNA by prior acid treatment has revealed bacterial nuclei as approximately spherical or dumbbell shaped often vesicular appearing elements Another type of staining reaction that has been prominent in the demonstration of nuclear elements of cells of higher organisms namely the binding of basic dyes by the acidic substances of the nucleus also has proved to be applicable to bacteria However since such basophily is exhibited not only by DNA but also by other acidic substances such as ribo-

nucleic acid (RNA) which is particularly abundant in the cytoplasm of bacteria it has been found necessary to pretreat bacteria with agents that remove RNA Exposure to ribo nuclease or to mild acid has proved to be suitable for the removal of the obscuring cyto plasmic RNA and has made possible a dis- tinct staining of bacterial nuclei with basic dyes (Robinow 1945) In electronmicro graphs the bacterial nucleus is indicated by regions of relative transparency (Fig 5)

Many bacteria possess only one nuclear body per cell but frequently 2 or more nuclei per cell can be discerned (Fig 7) Such multi nuclear state can be referred to the fact that under the conditions commonly employed for growth of bacteria, cellular and nuclear divi sion do not necessarily occur at the same rate The rate of nuclear division appears to be correlated with the rate of cellular growth Therefore in young actively growing cultures many multinuclear cells may be found because the nucleus may divide several times during the period of a single cell division (Figs 4 and 6) In older cultures uninuclear cells may predominate because general growth has now slowed down and cell division has had an opportunity to catch up with nuclear division Under the influence of certain physical and chemical agents that produce a selective in hibition of cell division an extreme separa tion of nuclear and cell divisions may occur and may lead to the formation of multinuclear filaments ultraviolet radiation and penicil lin are examples of such division inhibiting agents It may be added that naturally occur ring as well as experimentally produced modi fications of the nucleus cell ratio have been extremely useful in providing evidence for the function of the nucleus as a carrier of de terminants of hereditary characteristics (Wit kin 1951)

Little is known so far about the internal structure of bacterial nuclei The cytologic demonstration of intranuclear elements com parable with the organized chromosomes of higher organisms has remained in dispute Some investigators believe that they have identified chromosomelike bodies and have claimed that these undergo regular divisions similar to those known to occur in higher or ganisms Others have rejected such interpre tations However in view of the demonstrated relative regularity of the transmission of nu clear determinants of hereditary character istics from cell to cell (see p 61) some mechanism for the regular distribution of intranuclear elements can be presumed to exist Also other aspects of heredity in bac teria are consistent with the assumption that bacteria possess distinct intranuclear elements corresponding at least in function to the chromosomes of higher forms

Morphologic Aspects of Reproduction

In general bacterial cells multiply by binary

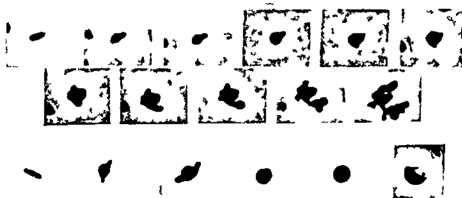


Fig 8 Phase contrast time lapse photomicrographs showing formation and multi plication of *E coli* protoplasts (0 to 4 hours) (*Top*) L-colony evolution from a single bacterium in penicillin + sucrose agar (*Bottom*) Successive stages of proto plast formation from an individual cell in liquid medium (penicillin + sucrose broth) (Lederberg J and St Clair J J Bact *75* 143 160)



FIG 9 Electronmicrograph of a longitudinal ultrathin section of *Bacillus cereus* in a late stage of sporulation × 40 500 (Chapman G B 1956 J Bact 71 348 355)

fission In this process cellular division preceded by nuclear division as discussed above is accomplished by the formation of a transverse cell wall Electronmicrographs (Fig 4) have revealed that the growth of transverse walls proceeds from the periphery toward the center of the cell much like the movement of a closing iris diaphragm In some cases it has been claimed that a *cytoplasmic* division presumably produced by the formation of new transverse cytoplasmic membranes may occur prior to the constrictive growth of the transverse wall After dividing cells do not necessarily separate from one another and thus various types of cell aggregates can arise (e g Figs 1 A C E 2 B) The mode of such aggregation depends in part upon the planes in which transverse wall formation occurs For example in the bacillary and spirillar forms cells tend to divide in a plane roughly perpendicular to the direction in which they grow among cocci the relative position of successive cross walls may shift with each division giving rise to regular (Fig 1 C) or irregular (Fig 1 A) clusters Following the separation of newly divided cells these may undergo different postfission movements relative to one another and particularly on solid media the resulting differences in cellular arrangement can have a decisive influence on colonial morphology

Some bacteria reproduce by budding and even members of bacterial species that normally reproduce by binary fission apparently can reproduce by budding when there is a cell wall deficiency (Fig 8) Such a deficiency can be produced as previously indicated (p 34) by temporary interference with wall synthesis or may arise spontaneously and persist in the case of the so called L forms (see p 73) In either case complex reproductive phenomena have been noted Some stable L forms have been reported to pass through complicated cycles involving giant cells that give rise to minute reproductive forms which will pass through filters with pore sizes of less than 1 μ These filterable forms sometimes have been claimed to undergo fusion prior to reproduction but despite current suspicions that they may be comparable with or related to protoplasts the significance of earlier observations regarding fusion of these forms remains enigmatic So far the only well documented case of sexlike fusion among eubacteria is that occurring in certain strains of *E coli* in which microscopically visible conjugation is associated with demonstrable transfers of genetic properties (see p 64)

SPORE FORMATION*

Some bacteria particularly bacilli and clostridia are capable of reproducing both vegetatively (i e by binary fission) and by endospore formation The formation of spores (Fig 9) occurs within a cytoplasmic region of the mother cell known as spore primordium which becomes surrounded by a highly refractile wall The shape and the position of the spore within the mother cell are relatively characteristic features for particular species Eventually mature spores are released from the confines of the mother cell by disintegration of the surrounding parental-cell material The spores also referred to as resting forms will germinate under suitable environmental conditions and give rise to typical vegetative cells Frequently there is a lag period (*dormancy*) between the exposure of spores to an environment suitable for germination and the actual onset of this process Such dormancy

* Reviewed by Halverson and Church 1957

can be of practical significance for example, it plays a role in the delay of toxin production in foods contaminated with spores of *Clostridium botulinum*

The composition of bacterial spores tends to differ considerably from that of vegetative cells The spores have a relatively low water content and at least in the cases so far examined contain (in free or combined form) a distinctive substance dipicolinic acid The most striking characteristic of bacterial spores is their great resistance to ordinarily adverse environmental conditions such as heat dehydration irradiation or exposure to toxic chemicals The basis for such increased resistance is not fully understood but presumably is related to the nature of the spore envelope the low degree of hydration of the spore, and its altered metabolic properties

By virtue of the enhanced resistance of spores their formation provides a mechanism for the survival of bacteria under environmental conditions that would be fatal to vegetative forms Thus dried spores are known to survive for a number of decades possibly even longer However sporulation is not necessarily dependent upon adverse environmental conditions it may occur also under conditions that permit vegetative multiplication therefore both spores and vegetative forms may coexist on occasion within the same environment It is also noteworthy that spore forming strains may give rise by mutation to asporogenous mutants

STAINING REACTIONS

Most of the morphologic elements described in the preceding section have been revealed with the aid of stains that possess a greater or lesser affinity for specific cell components The affinity of some of these stains depends upon the chemical nature of the cellular component with which they react Two staining procedures have attained particular significance as diagnostic tools in the identification and classification of bacteria the Gram stain and the acid fast stain

Gram Technic The Gram staining procedure consists of the following essential steps

1 Bacteria are stained with a basic triphenylmethane dye (usually of the methyl violet group)

2 They are mordanted by iodine in potassium iodide

3 They are thoroughly washed with a neutral organic solvent (in general ethanol or a mixture of ethanol and acetone)

4 They are counterstained with a dye of contrasting color (safranin for example)

The bacterial species which are decolorized by alcohol following treatment with iodine and take up the counterstain are referred to as gram negative whereas the gram positive species are those which retain the initial dye (crystal violet)

The mechanisms underlying the Gram stain still remain imperfectly understood In general the cells of gram positive species appear to be more acidic than those of the gram negative when measured by a variety of technics the "iso electric point' of the former (expressing the over all charge on the cell surface) is lower than that of the latter It seems not unlikely that gram positive cells contain an acidic substance probably located at the cell surface which is capable of retaining basic dyes even after treatment with iodine and subsequent washing with organic solvents At various times in the past a variety of substances have been suggested as responsible for gram positivity (Mitchell and Moyle 1950) including proteins phospholipids magnesium ribonucleate and most recently an unidentified phosphoric ester

Acid Fast Stain Tubercle bacilli and other mycobacteria are difficult to stain by the ordinary dyes Staining is achieved most readily by treating them with basic dyes in the presence of controlled concentrations of acid or alkali preferably with the help of heating Once stained under these conditions tubercle bacilli retain the dye even when washed for prolonged periods of time with ethanol acidified with strong acid a treatment which decolorizes all other bacterial types On the basis of these observations a number of method for the selective staining of acid fast bacteria have been developed The most commonly used of these is the Ziehl Neelsen technic which involves the following steps The preparation is stained with a mixture of fuchsin (a basic dye) and carbolic acid Although staining can take place slowly at room temperature it is much hastened by heating (for example by exposure to 100° C for a few minutes) This procedure stains all kinds of bacteria including endospores However subsequent thorough washing of the preparation with 95 per cent ethanol + 3 per cent mineral

acid removes the dye from all bacteria except the mycobacteria (in particular the pathogenic species) many types of spores also retain the fuchsin For convenience of observation it is usual to counterstain the organism, decolorized by acid alcohol with a dye of a contrasting color

Many theories invoking peculiar physicochemical properties of mycobacteria have been formulated to account for their staining properties Thus it is stated that the carbolfuchsin is more soluble in the cell constituents of mycobacteria than in the decolorizing agent It is of interest that in addition to mycobacteria a few types of bacterial endospores and of animal cell (helminth eggs hair) exhibit acid fastness Among constituents of mycobacteria only one substance has been found to retain fuchsin when treated by the Ziehl Neelsen technic It is mycolic acid a complex acid alcohol which occurs in the form of an ester in the tubercle bacillus Whether the presence of mycolic acid or like substances is sufficient to account for the acid fastness of mycobacteria remains however an unproved hypothesis

Although the Gram and the acid fast staining reactions were developed as empirical procedures and their mechanisms remain obscure it is interesting that they detect fundamental differences in the cellular structure of the different bacterial groups

Correlation Between Staining Characteristics and Biologic Properties

The Gram and the acid fast characters of bacteria have proved to be not only convenient for cytologic purposes but also valuable for purposes of classification since the classification based on staining properties corresponds to fairly well defined differences in chemical and biologic properties (see also p 53) There fore it appears worthwhile to review briefly some of the characteristics which appear to be correlated with staining properties

Measurements of electrophoretic mobilities and of the ability to retain basic dyes indicate a marked preponderance of acid over basic groups in the gram positive bacteria and have led to the statement that these organisms possess over all isoelectric points ranging from pH 2 5 to 4 0 In general gram positive differ from gram negative organisms in their susceptibility toward certain antibacterial agents for example the former organisms are usually much more susceptible to the inhibiting effect of basic dyes anionic detergents penicillin gramicidin bacitracin subtilin etc however there are important exceptions to this rule such as the great susceptibility of gonococci and meningococci to penicillin

The isoelectric points (as described above) of the gram negative organisms are clustered between pH 4 5 and 5 5 i e less on the acid side than in the case of gram positive bacteria When smooth gram negative organisms (see p 74) are extracted by a variety of reagents (trichloracetic acid diethylene glycol phenol aqueous pyridine etc) they yield in solution phospholipid polysaccharide protein complexes known as *O antigens* (see p 75) and endotoxins These determine the immunologic specificity of the strain their toxicity is not readily destroyed by high temperatures In spite of many attempts complexes with similar antigenic and toxic properties have not yet been recovered from gram positive bacteria As already mentioned most gram negative rods and vibrios are much more resistant to certain antibacterial agents than the gram positive species On the other hand they are extremely susceptible to the bactericidal effects of specific immune serum in the presence of complement both in vitro and in vivo

Acid fast bacteria are difficult to stain and once stained equally difficult to decolorize These organisms are characterized by extremely high concentrations of a variety of lipids which may reach up to 30 per cent of the total weight of the cell in the pathogenic species It is certain that the high lipid concentration is responsible for the hydrophobic character of the cell surface of mycobacteria and for their peculiar mode of growth on the surface of aqueous media It is also known that the different lipids elicit characteristic types of tissue response in the invaded host playing a dominant part in the histopathologic picture of tuberculosis As a group acid fast bacteria differ from the nonacid fast species in their behavior toward antibacterial agents Although they are resistant to certain antiseptics they exhibit marked susceptibility to others which are only poorly active against the ordinary nonacid fast species Special mention must be made of the striking resistance of tubercle bacilli to strong acids and alkalis a property which is widely used for the selective elimination from suspected pathologic material of contaminating nonacid fast

bacteria which interfere with the bacteriologic diagnosis of tuberculosis

BACTERIAL PHYSIOLOGY*

Bacteria as Typical Cells

A vast body of evidence sustains the con clusion that bacteria and other forms of life have various fundamental features in com mon As we have seen the bacteria are cells which like other cells have nuclear and cyto plasmic components and are bounded by cell envelopes Perhaps even more suggestive than this correspondence in gross morphologic traits are resemblances in structure at the molec ular level For instance bacterial and other types of cells contain not only similar small molecules (such as amino acids purines pyr imidines sugars lipids and vitamins) but also similar macromolecules (such as proteins nucleic acids and polysaccharides)

The similarities in gross and molecular morphology between bacteria on the one hand and the rest of the living world on the other are strikingly reflected in the results of *physi ologic* investigations Thus the *functioning* of bacteria as living organisms has pronounced parallels with that of other cells and the notion of a common basic blueprint for all forms of life is impressively documented It must be stressed however that despite the basic similarities noted bacteria do have nu merous important distinguishing features which are indicative of special physiologic mechanisms Some such mechanisms will be reviewed in a subsequent section

Some Aspects of the Cellular Growth Process The physiology of bacteria is perhaps approached best from considerations of cel lular growth which in turn is intimately linked to cell multiplication In this context growth may be viewed as an increase in the characteristic' matter of a cell Like many other forms bacteria can grow and multiply at the expense of raw materials (nutrients) of relatively simple molecular structure The chemical transformation of such small mole cules involves almost without exception catal ysis by macromolecules (enzymes) There fore, the cells must be able to synthesize not only comparatively small molecules but also macromolecules such as proteins Since en zymes in general catalyze single steps, for example the making or breaking of a chemi cal bond it seems reasonable to expect that the synthesis of an enzyme itself (which may contain thousands of distinctive peptide bonds) cannot proceed by enzymatic catalysis in the ordinary sense It is usually assumed that the synthesis of an enzyme depends on a specific multifunctional macromolecular catalyst, termed *template* Therefore the syn thetic activities of bacterial cells as those of other cells must include the production of templates enzymes and small molecules In addition the synthesis of certain other macro molecular substances commonly found in liv ing cells must be accomplished Since the templates control the formation of the cell s catalysts they have a determining influence on the biochemical and physiologic activities of the cell To the extent that templates are replicable they can pass on a code of the cell s character from generation to generation and thus provide for genetic continuity

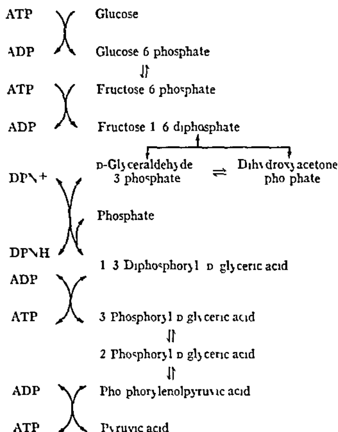
Energy Requirement for Growth Clearly growth can occur only if the cell s synthetic activities are closely co-ordinated in space and in time The production of cell material from simple nutrients and the concomitant emer gence of highly organized structures of course require an input of energy Living cells gen erally depend on one of two sources of energy chemical energy available from oxidation reduction reactions or radiant energy (light) Most bacteria obtain their energy from those oxidation reduction reactions in which *organic* oxidizable substrates (e g glucose) are uti lized Such bacteria are called *organotrophs* Certain other bacteria that use inorganic oxi dizable substrates are *lithotrophs* It should be noted that organotrophy is also found for instance in animals and lithotrophy in plants In the oxidation reduction reactions the oxi dizing agents may be (1) molecular oxygen (2) other inorganic substances or (3) organic compounds When oxygen is used we speak of *respiration* when organic substances func tion as oxidizing agents (and also as oxidiz able material) we speak of *fermentation* (although the latter term is not always em ployed in this strict sense) As will be illus

* Detailed recent discussions of bacterial physiology can be found in Werkman and Wilson 1951 La manna and Mallette 1953 Oginsky and Umbreit 1954 Kluyver and van Niel 1956

trated in a sub equent section certain of the bacterial mechanisms of respiration and fer mentation have their counterparts in other forms of life

The Uptake of Nutrients In general organotrophic bacteria use their organic substrates not only as a means of obtaining energy but also as the main source of the element carbon In addition the elements hydrogen oxygen nitrogen sulfur phosphorus potassium magnesium and certain others are required for growth and must be supplied in a suitable chemical form (see p 56) The uptake of at least some nutrients is an active energy requiring process which may be specific for certain molecules or groups of molecules Furthermore many substances once having penetrated can be maintained within the cells often at relatively high concentrations and against large concentration gradients The characteristics of nutrient uptake are of course dependent on the properties of the cell envelope (see p 30) In the case of many living forms including the bacteria only substances of low molecular weight usually enter the cell Some bacteria produce extracellular enzymes capable of breaking down large molecules (such as pro-

THE GLYCOLYTIC PATHWAY



Abbreviations

ATP = adenosine triphosphate
ADP = adenosine diphosphate
DPN+ and DPNH = oxidized and reduced diphosphopyridine nucleotide

teins or polysaccharides) into smaller units which are then utilizable by the cells Once the nutrient materials have become available to the cell they are metabolized, i e they are subjected to the numerous chemical reactions associated with the maintenance and the reproduction of the organism

Small Molecule Metabolism Bacteria share with other cells the possession of certain central chemical mechanisms for the metabolism of small molecules These mechanisms furnish at one and the same time necessary energy and specific intermediates for the synthetic activities of the cell One important and widely utilized small molecule substrate that can serve as an energy and as a carbon source for many bacteria and other organisms is glucose Accordingly small molecule metabolism will be illustrated below with a succession of pathways leading to various essential cell components from glucose as the initial substrate

THE GLYCOLYTIC PATHWAY * In general the utilization of glucose involves the formation of pyruvate as a pivotal metabolite A sequence of reactions known as the glycolytic pathway which leads from glucose to pyruvate is represented in the accompanying diagram This pathway is widespread in nature it is found in bacteria and other micro organisms as well as in plants and animals Several of the enzymes of the glycolytic pathway act in conjunction with coenzymes (see p 49) which are more or less complex organic substances whose molecular weight is low compared with that of the enzymes In some cases metal ions are required as cofactors

The glycolytic pathway has a number of *noteworthy features among them that all the* intermediates involved are phosphorylated In the first phase of glycolysis glucose is converted to the 6 carbon compound fructose 1 6 diphosphate which is cleaved to two interconvertible 3 carbon compounds (triosephosphates) namely D-glyceraldehyde 3 phosphate and dihydroxyacetonephosphate The D glyceraldehyde 3 phosphate is then further metabolized, and so is the dihydroxyacetonephosphate after conversion to D-glyceraldehyde 3 phosphate The next step is the dehydrogenation of D-glyceraldehyde 3 phosphate to 1 3 diphosphoryl D glycerate In the last phase of glycolysis the latter compound is converted to pyruvate

* For a recent discussion of this pathway in various organisms see Fruton and Simmonds 1953 Neilands and Stumpf 1955

The last phase of glycolysis is particularly interesting because in it a portion of the chemical energy which originally resided in the glucose molecule is "trapped for use in the energy requiring processes of the cell The trapped energy is preserved as bond energy in a key chemical compound, adenosine triphosphate (ATP), which is formed from adenosine diphosphate (ADP) and inorganic phosphate In the formation of ATP from ADP a pyrophosphate bond of acid anhydride character is produced This type of bond has a high energy content compared with the ester type bond found for example in glucose 6 phosphate * ATP is a versatile compound which, through participation in numerous enzymatic reactions can make its stored energy available for the performance of chemical work The utilization of ATP for synthetic purposes will be illustrated in the section on macromolecule metabolism (p 49) but there are also many well known instances of the participation of ATP in the synthesis of small molecules

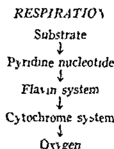
The generation and the trapping of high energy phosphate bonds can be followed in the diagram on page 41 the glycolytic pathway Generation occurs in the formation of 1 3 diphosphoryl D glycerate and phosphorylenolpyruvate which contain one such bond per molecule Trapping occurs when each of these two compounds transfers a portion of its energy in a reaction in which ADP is converted to ATP Since 1 molecule of glucose gives rise to *2 molecules of pyruvate* 4 molecules of ATP are formed per molecule of glucose used However 2 molecules of ATP are consumed in the conversion of 1 molecule of glucose to fructose 1 6 diphosphate accordingly the net yield from glycolysis is 2 molecules of ATP per molecule of glucose Although the glycolytic pathway is widely distributed in nature it is not the only mechanism that permits the formation of pyruvate from glucose another such mechanism in

* Bond energy in this biochemical context has a special meaning in speaking of high energy bonds biochemists refer to the relatively large negative *standard free energy changes* associated with the hydrolytic cleavage of these bond

volves the so called hexosemonophosphate shunt (Gunsalus et al 1955)

FERMENTATION When glycolysis proceeds under conditions of fermentation (see p 40) pyruvate production (which involves a dehydrogenative i e oxidative step) must be balanced by the formation of organic substances more reduced than pyruvate (This balancing is necessary since the occurrence of any oxidation depends on the occurrence of an equivalent reduction) The immediate electron acceptor in the dehydrogenative step is diphosphopyridine nucleotide (DPN see p 48) which however acts only catalytically (with formation of reduced pyridine nucleotide) Therefore the electrons must be transferred to an ultimate acceptor In one type of fermentation (*homofermentative lactic fermentation*) pyruvate itself functions as electron acceptor and is converted to lactate The production of lactate through glycolysis is characteristic of the lactic acid bacteria and occurs in many other cells including those of mammalian muscle tissue In other types of fermentation pyruvate is metabolized to other products that act as electron acceptors (see p 54) In the *alcoholic fermentation* for instance pyruvate is decarboxylated to acetaldehyde which is reduced to ethanol Alcoholic fermentation is characteristic of yeasts it also occurs in some bacteria and other organisms

RESPIRATION When glycolysis proceeds under conditions of respiration (see p 40) rather than of fermentation the formation of pyruvate does not have to be balanced by the net reduction of *organic* substances but can be balanced ultimately by the reduction of molecular oxygen to water As indicated above the requirement for an ultimate electron acceptor arises from the fact that glycolysis includes a dehydrogenative step which leads to intermediate formation of reduced pyridine nucleotide The latter in turn must be dehydrogenated for glycolysis to continue The dehydrogenation (oxidation) of reduced pyridine nucleotide and the ensuing reduction of oxygen involve a complex transport system in which electrons are transferred from the nucleotide to the oxygen The resulting negatively charged oxygen then reacts with hydrogen ions to yield water The transport system may include flavoproteins and a sequence of cytochromes one of which cytochrome a_3 (cytochrome oxidase) is able to transfer electrons to molecular oxygen The flavoproteins and the cytochromes are characterized, respectively by containing flavins and iron porphyrins (see p 49) The respiratory process described (which however, is not the only known process of its kind) is summarized in the accompanying diagram



Simplified scheme of electron transfer from a substrate to molecular oxygen

The coaction of the glycolytic and respiratory processes makes possible the continued conversion of glucose to pyruvate (without necessitating the formation of reduced derivatives of pyruvate) Pyruvate in turn can be converted to carbon dioxide and water through another respiratory mechanism (the citric acid cycle) which is of widespread if not ubiquitous occurrence among living cells including the bacteria Thus metabolic pathways for the complete oxidation of glucose are available The manner in which cells use these pathways for the channeling of energy and the production of biosynthetic intermediates will be discussed in subsequent sections

THE CITRIC ACID CYCLE * Prior to its complete oxidation to carbon dioxide and water through the metabolic system known as the citric acid cycle pyruvate is subjected to oxidative decarboxylation In this partial oxidation carbon dioxide is liberated from the carboxyl group of pyruvate and the remaining 2 carbon moiety appears as an acetyl derivative of coenzyme A (see p 48) This derivative enters the citric acid cycle

In the first step acetyl coenzyme A reacts with oxaloacetate to yield citrate with th

* For details consult Fruton and Simmonds 1953 Roberts et al., 1955

teins or polysaccharides) *into smaller units* which are then utilizable by the cells Once the nutrient materials have become available to the cell they are metabolized i e, they are subjected to the numerous chemical reactions *associated with the maintenance and the re* production of the organism

Small Molecule Metabolism Bacteria share with other cells the possession of certain central chemical mechanisms for the metabolism of small *molecules These mechanisms furnish* at one and the same time necessary *energy* and specific intermediates for the synthetic activities of the cell One important and widely utilized small molecule substrate that *can serve as an energy and as a* carbon source for many bacteria and other organisms is glucose Accordingly small molecule metabolism will be illustrated below with a succession of pathways leading to various essential cell com *ponents from glucose as the initial substrate*

The Glycolytic Pathway * In general the utilization of glucose involves the formation of pyruvate as a pivotal metabolite A sequence of reactions known as the glycolytic *pathway, which leads from glucose to pyruvate* is represented in the accompanying diagram This pathway is widespread in nature it is found in bacteria and other micro organisms as well as in plants and animals Several of *the enzymes of the glycolytic pathway act in* conjunction with coenzymes (see p 49) which are more or less complex organic substances whose molecular weight is low compared with that of the enzymes In some cases metal ions *are required as cofactors*

The glycolytic pathway has a number of noteworthy features among them that all the intermediates involved are phosphorylated In *the first phase of glycolysis glucose is con* verted to the 6 carbon compound, fructose 1 6 diphosphate, which is cleaved to two inter convertible 3 carbon compounds (triosepho *phates) namely* D-*glyceraldehyde* 3 phosphate and dihydroxyacetonephosphate The D-glycer aldehyde 3 phosphate is then further metabo lized and so is the *dihydroxyacetonephosphate after* conversion to D glyceraldehyde 3 phos phate The next step is the dehydrogenation of D-glyceraldehyde 3 phosphate to 1 3 di *phosphoryl* D *glycerate In the last* phase of glycolysis the latter compound is converted to pyruvate

The last phase of glycolysis is particularly interesting because in it a portion of the chem ical energy which originally resided in the glucose molecule is "trapped for use in the *energy requiring processes of the cell* The trapped energy is preserved as bond energy in a key chemical compound, adenosine tri phosphate (ATP), which is formed from ade nosine diphosphate (ADP) and inorganic phosphate *In the formation of ATP from* ADP, a pyrophosphate bond of acid anhydride character is produced This type of bond has a high energy content compared with the ester type bond *found* for example in glucose 6 *phosphate* * ATP is a versatile compound which, through participation in numerous enzymatic reactions can make its stored energy available for the performance of chem ical work *The utilization of ATP for synthetic* purposes will be illustrated in the section on macromolecule metabolism (p 49) but there are also many well known instances of the participation of ATP in the synthesis of small *molecules*

The generation and the trapping of high *energy phosphate bonds can be followed in* the diagram on page 41 the glycolytic pathway Generation occurs in the formation of 1 3 diphosphoryl D glycerate and phos*phorylenolpyruvate which contain one such* bond per molecule Trapping occurs when each of these two compounds transfers a po tion of its energy in a reaction in which ADP *is converted to* ATP Since 1 molecule of glucose gives rise to 2 molecules of pyruvate 4 molecules of ATP are formed per molecule of glucose used However, *2 molecules of ATP* are consumed in the conversion of 1 molecule of glucose to fructose 1 6 diphosphate ac cordingly, the net yield from glycolysis is 2 molecules of ATP *per molecule of glucose* Although the glycolytic pathway is widely distributed in nature it is not the only mecha nism that permits the formation of pyruvate *from glucose* another such mechanism in

* For a recent discus ion of this pathway in various organisms ee Fruton and Simmond 1953 Neilands and *Stumpf* 1955

* Bond energy in *this biochemical context* has a special meaning in speaking of high energy bonds biochemists refer to the relatively large negative *standard free energy changes* associated with the hydrolytic cleavage of these bonds

mediate formation of fumarate and malate finally yields oxaloacetate which is then ready for another turn of the cycle The citric acid cycle is thus capable of accepting a 2 carbon fragment and in one turn of liberating 2 molecules of carbon dioxide with the regeneration of the 4 carbon compound oxaloacetate (which originally had accepted the 2-carbon fragment) However it is not implied that the regenerated and original oxaloacetate correspond atom for atom

The citric acid cycle taken together with the sequence of glycolysis and the oxidative decarboxylation of pyruvate can account for the complete oxidation of glucose The cycle also catalyzes the oxidation of any other substance that gives rise to the acetyl moiety of acetyl coenzyme A The function of the cycle in supplying intermediates for biosynthetic purposes is presented in another section

In the cycle proper there are 4 dehydrogenative (oxidative) reactions whose substrates are isocitrate α ketoglutarate succinate and malate In the respiratory operation of the cycle these oxidative steps are balanced by the reduction of molecular oxygen to water through the mediation of electron transport systems

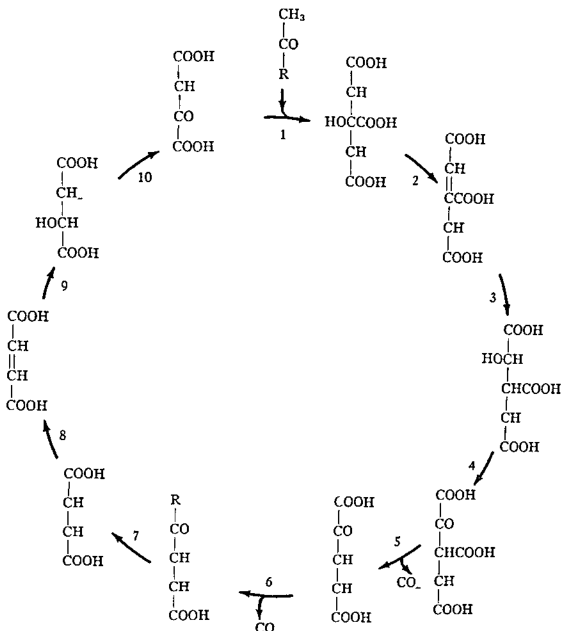
Oxidative Phosphorylation and Energy Relationships * Coupled with the stepwise transport of electrons from various oxidizable substrates to oxygen is a most important cellular process known as oxidative phosphorylation In this process the transfer of electrons is accompanied by the production of high energy phosphate bonds which can be stored as ATP However the electron transport can take place without a *net formation* of ATP This separation of oxidation from phosphorylation can be brought about by *uncoupling agents* such as 2 4 dinitrophenol Oxidative phosphorylation has been demonstrated in a wide variety of cells including bacteria

In view of the numerous pyridine nucleotide dependent reactions known to occur oxidative phosphorylation associated with electron transport from reduced pyridine nucleotide to oxygen (with formation of the oxidized form of pyridine nucleotide) is of particular importance Experiments with mammalian preparations have revealed that in the oxidation of reduced pyridine nucleotide 3 molecules of ATP can be formed (from ADP) per atom of oxygen reduced This yield of ATP is relatively high it represents about 75 per cent of the highest possible yield predicted from thermodynamic measurements In another instance of oxidative phosphorylation namely that accompanying the dehydrogenation of succinate to fumarate a ratio of about 2 molecules of ATP produced per atom of oxygen reduced has been demonstrated Oxidative phosphorylation is seen to be an efficient means of trapping energy in respiratory metabolism and accounts for a substantial portion of the energy that becomes available to the cell through the complete oxidation of glucose With the aid of the above mentioned figures for the yield of ATP in oxidative phosphorylation (and with certain assumptions) the efficiency of the trapping of energy can be estimated readily The *maximum* energy that can become available to cells for useful work through the oxidation of glucose to carbon dioxide and water has been calculated from thermodynamic data to be about 690 kilocalories per gram mole of glucose The energy stored in 1 gram mole of ATP in terms of the hydrolysis of the latter to ADP is approximately 8 kilocalorie Therefore a 100 per cent efficient trapping would mean the generation of 690 divided by 8 or roughly 86 molecules of ATP formed from ADP per molecule of glucose oxidized In the complete oxidation of glucose the first oxidative step occurs at the triosephosphate level There are 6 oxidative steps in all 5 of them are pyridine nucleotide dependent and give an oxidative phosphorylation yield of 15 molecules of ATP per molecule of *triosephosphate* The remaining oxidative step (whose substrate is succinate) accounts for 2 molecules of ATP The total oxidative phosphorylation yield per molecule of the *hexose* glucose hence is 2 times 17 or 34 molecules of ATP To this figure must be added 2 molecules of ATP from the operation of the glycolytic pathway itself and 2 molecules of ATP formed in connection with the conversion of succinyl coenzyme A to succinate (in the citric acid cycle) Therefore the grand total is a yield of 38 molecules of ATP produced per molecule of glucose oxidized and the efficiency of the over all process is 38 divided by 86 times 100 i e about 44 per cent From the untrapped fraction of the energy heat is produced

The yield of ATP (38 molecules) resulting from the respiratory process may be compared and contrasted with the yield of ATP (2 mole

* Reviewed by Wood 195 Neiland and Stumpf 1955 Pinchot 1953

CITRIC ACID CYCLE



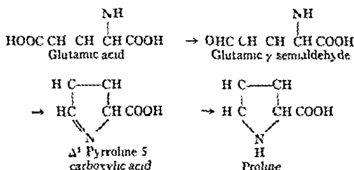
In the clockwise operation of the cycle shown one molecule of a two carbon fragment is introduced as acetyl coenzyme A and two molecules of carbon dioxide are produced per turn of the cycle R represents the coenzyme A moiety No implications as to the reversibility of the individual reactions are intended The products of reactions 1 to 10 are, respectively citric acid cis aconitic acid isocitric acid oxalosuccinic acid α keto glutaric acid succinyl coenzyme A succinic acid fumaric acid malic acid oxaloacetic acid

concomitant regeneration of coenzyme A The 6 carbon compound citrate is converted in a step wise manner to the 5 carbon compound α ketoglutarate the sixth carbon atom is eliminated as carbon dioxide α Ketoglutarate also loses a molecule of carbon dioxide in this case through oxidative decarboxylation with formation of succinyl coenzyme A This acyl derivative of coenzyme A in the presence of ADP and inorganic phosphate gives rise to the 4 carbon compound succinate and also to ATP and coenzyme A Succinate with inter

cells can utilize keto acids which are derivable from glycolysis or the citric acid cycle for the synthesis of glutamic and aspartic acids and α alanine. Glutamate can give rise to certain other amino acids (including ornithine, citrulline, arginine and proline) without the loss of its amino group. The glutamate to proline pathway which appears to occur in bacterial, plant and animal cells is shown schematically.

The salient features of small molecule biosynthesis as exemplified by the formation of proline from glucose (see the accompanying diagram) are the step wise enzyme catalyzed reactions arrayed in linear, cyclic or branched pathways. These pathways may be conceived not only in terms of the sequence of intermediates involved but also in terms of the participating enzymes and their spatial organization within the cell. The same general features are observable in the biosynthesis of other

GLUTAMATE TO PROLINE PATHWAY



A SYSTEM OF PATHWAYS LEADING FROM GLUCOSE TO PROLINE

Glucose
↓ Via glycolytic pathway
Pyruvic acid —— Reductive CO addition → Malic acid
Pyruvic acid ↓ Oxidative decarboxylation → Acetyl coenzyme A
Malic acid ↓ Dehydrogenation → Oxaloacetic acid
Acetyl coenzyme A + Oxaloacetic acid ↓ Via part of citric acid cycle
α Ketoglutaric acid
↓ Reductive amination
Glutamic acid
↓ Glutamate to proline pathway
Proline

cules) obtained in the fermentation of 1 molecule of glucose to lactic acid This fermentative mechanism thus furnishes, per molecule of glucose consumed, very much less useful energy than does the respiratory process described

BIOSYNTHESIS OF SMALL MOLECULES * In the preceding sections a series of mechanisms has been presented by which glucose can give rise to pyruvate and eventually to carbon dioxide and water The pathways involved have been discussed mainly from the standpoint of energy yield However they have another vital function, namely the supplying of intermediates for biosynthetic activities

As pointed out above the citric acid cycle can perform as a catalytic system for the oxidation of 2-carbon fragments supplied as acetyl coenzyme A In this catalytic operation of the cycle oxaloacetate accepts the 2 carbon fragment and is regenerated with the intermediate formation of several 6 5 and 4 carbon compounds It is clear that any removal of intermediates from the cycle would prevent the regeneration of the full amount of oxaloacetate and would lead to a progressive slowing down of the cycle action Hence if the cycle is to provide precursors for biosynthesis i e if it is to permit a steady withdrawal of cycle intermediates it must receive not only an influx of acetyl coenzyme A but also of oxaloacetate The origin of the acetyl moiety of acetyl coenzyme A from pyruvate metabolism has already been discussed Pyruvate can also be involved in the production of a steady flow of oxaloacetate for example through the combination of the following two reactions in the first reaction (which is mediated by the so called malic enzyme) pyruvate and carbon dioxide react in the presence of reduced pyridine nucleotide to yield malate and in the second reaction (catalyzed by malic dehydrogenase) malate and pyridine nucleotide interact with the formation of oxaloacetate

* For a recent review of methods for the analysis of biosynthetic pathways see Vogel and Bonner 1958

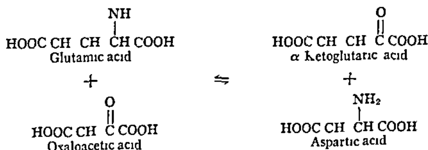
Experiments with radioactive tracers have shown that the citric acid cycle can indeed play a major role in biosynthesis One of the biosynthetic intermediates that the cycle supplies is α ketoglutaric acid This compound and its derivative glutamic acid are highly important, versatile metabolites

The production of glutamic acid from α ketoglutaric acid is particularly significant since it represents for many bacterial and other cells the step that is responsible for the conversion of inorganic nitrogen (in the form of ammonium ions) to organic nitrogen The reaction involved is mediated by the enzyme glutamic dehydrogenase in the presence of reduced pyridine nucleotide

Among the numerous functions of glutamate is the transfer of its amino group and hence of its nitrogen to other cell components One type of reaction that permits such a transfer is known as *transamination* (see Meister 1957) An instance of transamination is illustrated here In this case the amino acid glutamic acid and the keto acid oxaloacetic acid react (in their respective ionic forms) to yield the keto acid α ketoglutaric acid and the amino acid, aspartic acid In an analogous transamination reaction glutamate can donate its nitrogen to pyruvate with the formation of the amino acid α alanine Enzymes catalyzing transaminations are called transaminases in a number of instances they have been found to depend on pyridoxal phosphate (see p 48) as coenzyme

Thus we have seen how bacterial and other

A TRANSAMINATION



amino acids and indeed (as far as is known) in the formation of all other required small molecules such as purines pyrimidines lipids pigments and vitamins

The vitamins serve as a particularly graphic illustration of the biochemical and physiologic unity of the various forms of life They were originally characterized as growth factors for animals and were subsequently found to be required for the growth of certain bacteria (see p 57) and many other organisms On the other hand numerous bacteria and other forms do not require an external supply of vitamins but are capable of synthesizing them The generalization can be made then that all forms of life seem to depend on certain vitamins (whether furnished exogenously or synthesized endogenously) *In general the role* of vitamins in metabolism is associated with the biosynthesis of coenzymes The structures of some coenzymes which have been considered in this chapter are illustrated here In this diagram the function of certain vitamins (nicotinamide pantothenic acid riboflavin pyridoxal) as coenzyme moieties is indicated

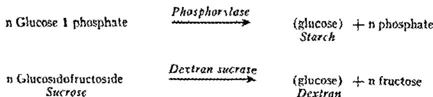
To carry out their catalytic activities coenzymes generally become linked to their respective apoenzymes (which are the protein moieties of enzymes) The complete functional enzyme i e apoenzyme plus coenzyme is termed holoenzyme Coenzymes conceived as portions of holoenzymes are usually called prosthetic groups Prosthetic groups differ in their tendency to dissociate from their respective holoenzymes For example diphosphopyridine nucleotide dissociates relatively readily this dissociability is of value in the metabolic linking of several different pyridine nucleotide dependent enzymatic reactions On the other hand iron porphyrin holoenzymes have a much lesser tendency to dissociate

Among the iron porphyrin enzymes are the catalases and the peroxidases (collectively known as hydroperoxidases) and the cytochromes (see p 43) The hydroperoxidases participate in reactions in which a molecule of an oxidizable substrate H_2A and a molecule of hydrogen peroxide H_2O_2 yield (in an over all sense) the oxidized compound A and 2 molecules of water In the particular case where H_2A is H_2O_2 we have H_2O_2 plus H_2O_2 yielding O_2 plus $2H_2O$ a reaction which represents the decomposition of hydrogen peroxide with formation of oxygen and water and is characteristically mediated by catalases Relatively high catalase activity occurs in various aerobic bacteria (and in many other kinds of cells) and the presence (or the absence) of this activity is of value in bacterial classification

Macromolecule Metabolism As illustrated in the preceding section the metabolism of small molecules depends immediately on at least one class of macromolecules namely the enzymes Let us now turn to an examination of the salient features of the metabolism in particular the biogenesis of various macromolecules

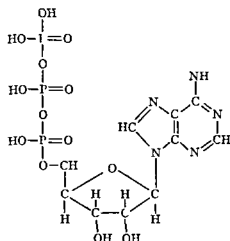
Bacterial macromolecules as well as those of other forms of life range in molecular weight from say a few thousand into the millions The macromolecules of biologic origin may be regarded as consisting of one or more types of low molecular units joined together to yield large molecules of greater or lesser complexity or distinctiveness At one end of the scale of distinctiveness are for example certain polysaccharides which are characterized by a single kind of repeating

SYNTHESIS OF CERTAIN POLYSACCHARIDES

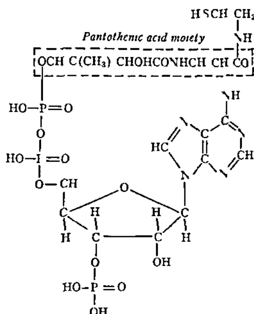


Synthesis of certain polysaccharides Phosphorylases are enzymes that occur in bacteria as well as in plant and animal tissues Dextran sucrase activity is found in various organisms e g in Leuconostoc mesenteroides

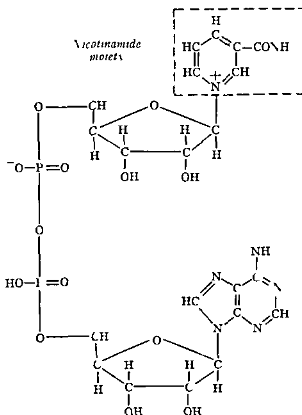
SOME COENZYMES



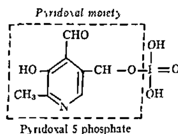
Adenosine 5 triphosphate (ATP)



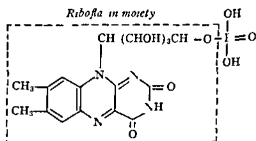
Coenzyme A



Diphosphopyridine nucleotide (DPN)



Pyridoxal 5 phosphate



Flavin phosphate

amino acids and indeed (as far as is known) in the formation of all other required small molecules such as purines, pyrimidines, lipids, pigments and vitamins.

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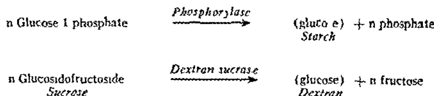
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Bacterial macromolecules, as well as those of other forms of life, range in molecular weight from, say, a few thousand into the millions. The macromolecules of biologic origin may be regarded as consisting of one or more types of low molecular units joined together to yield large molecules of greater or lesser complexity or distinctiveness. At one end of the scale of distinctiveness are, for example, certain polysaccharides which are characterized by a single kind of repeating

SYNTHESIS OF CERTAIN POLYSACCHARIDES



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unit (e g , a glucose residue) and a single type of bond between such units Polysaccharides of bacterial plant, or animal origin, such as starches or dextrans are synthesized in an apparently step wise manner from suitable sugar derivatives as illustrated here Macro molecules of such a relatively low degree of distinctiveness are synthesized by enzymes much like those that mediate the synthesis of small molecules At the other end of the scale of distinctiveness are the proteins, including the enzymes which generally are composed of some 20 different amino acids linked in such a manner that each protein species comprises an apparently characteristic array of several dozen, hundreds or even many thousands of individual amino acids

PROTEIN SYNTHESIS * As noted earlier (see p 40) the specific arrays of structural units present in proteins presumably cannot be pro duced through the agency of enzymes such as those that catalyze the formation of small molecules Rather the biogenesis of proteins is thought to depend on multifunctional cata lysts termed templates'

Templates appear to be concerned with the emergence of the characteristic amino acid sequence (patternization) of the polypep tide chains Patternization however is not the only event in protein biogenesis The entire process involved may be viewed as consisting of the following phases

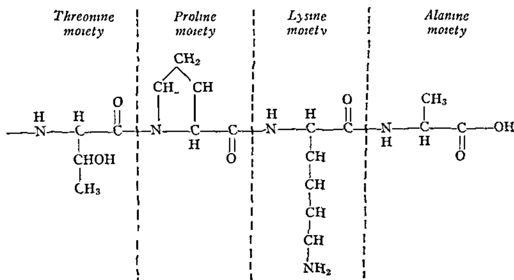
1 Biosynthesis (or uptake) of amino acids
2 "Activation of amino acids
3 Transfer of activated amino acids to templates
4 Polypeptide formation from active pat ternized amino acids
5 Separation of polypeptide chains from templates
6 Final shaping of proteins

The first two phases deal with small molecule precursors of protein the next two phases with the synthesis of the polypeptide chain and the last two phases with the pro duction of the 'finished' protein molecule

Enzymes capable of activating the carboxyl group of certain amino acids have been ob tained from bacteria and other micro organ isms as well as from animal and plant tissues Activation at least in one form results when an amino acid, ATP, and an appropriate enzyme interact to produce a mixed acid an hydride of the amino acid and adenosine monophosphate (with elimination of pyro phosphate) Activated amino acid residues then appear to be transferred to this or that template where they may be presumed to exist transiently as carboxyl activated derivatives Polypeptide formation would then come about

* See Lipmann 1956 Hoagland et al 1956 DeMoss and Novelli 1955

TERMINAL PORTION OF A POLYPEPTIDE CHAIN



through the orderly reaction of the activated carboxyl group of each amino acid residue with the amino group of the immediately adjacent residue It must be pointed out that the above picture of polypeptide formation is inferential in various respects and that some investigators hold that the synthesis of protein polypeptides may involve the participation of oligopeptides as precursors A portion of a polypeptide chain is shown here

In the last two phases of protein synthesis newly formed polypeptide chains are thought to dissociate from their respective templates and to undergo final shaping The latter process may be more or less complex and may involve for instance the aggregation of polypeptide chains and their folding to give the finished 3 dimensional protein

Small Molecule Control of Protein (Enzyme) Synthesis The last two phases namely the separation of polypeptide chains from templates and the final shaping of proteins have been implicated in certain control mechanisms that can regulate the production of some enzyme proteins One such mechanism enzyme induction (or enzymatic adaptation see reviews by Monod and Cohn 1952 Spiegelman and Campbell 1956) permits an enhanced synthesis of certain enzymes in response to specific small molecules such as the *substrates* (or chemically related substances) of the respective enzymes (see also p 62) Enzyme induction has been observed in microorganisms including bacteria as well as in animal and plant tissues For example some bacteria that under various cultural conditions produce relatively low levels of an enzyme capable of hydrolyzing the sugar lactose will synthesize much larger amounts of the enzyme if cultivated in the presence of lactose The enzyme thus synthesized brings about the cleavage of the lactose into its component monosaccharides which become available to the metabolic machinery of the organisms While enzyme induction is thus seen to be a valuable regulatory device it is possible under certain conditions to induce the formation of enzymes that are of no apparent advantage to the cells involved

Another regulatory device that can affect enzyme formation is enzyme repression (Vogel 1957) This device can bring about a decrease in the rate of synthesis of certain enzymes in response to specific small molecules such as the end products (or chemically related substances) of the biosynthetic pathways in which the respective enzymes participate When for instance one of certain amino acids is added to susceptible bacterial cultures there will result a lowering of the levels of one or more enzymes concerned with the *endogenous* formation of this amino acid (with concomitant utilization of the *exogenous* supply of this amino acid) It would appear then that enzyme induction and enzyme repression are complementary control mechanisms In either case the cell tends to form enzymes when they are needed and tends not to form enzymes when they are not needed

Nucleic Acid Synthesis * While enzyme induction and repression undoubtedly play important roles in the physiology of bacteria and other cells perhaps the most striking directive influence in protein biogenesis is that thought to be exerted by the templates The intriguing question of the chemical nature of templates has not yet been answered conclusively However there are good grounds for the belief that the nucleic acids (see p 67) contain within them the information † (i e the patternization code for protein synthesis) that is expected of templates

The nucleic acids are macromolecules whose small molecule building blocks are the nucleotides There are two major types of nucleic acids deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) depending on the sugar moiety (deoxyribose or ribose) of the respective component nucleotides The structures of some nucleotides and of a portion of a nucleic acid chain are shown here The way in which nucleic acids may store information for protein synthesis and a further discussion of nucleic acid structure can be found on p 67 Many investigators believe that such information can be contained in either DNA or RNA The possibility exists that DNA is a primary template from

See reviews by Ochoa and Heppel 1957 Kornberg 195

† The term information is used here and also on p 53 in reference to molecular structure the same term has also been employed in relation to hereditary determinants (see p 61)

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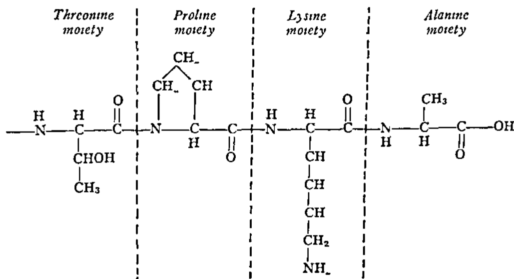
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6 ' Final shaping of proteins

The first two phases deal with small molecule precursors of protein the next two phases with the synthesis of the polypeptide chain, and the last two phases with the pro duction of the finished protein molecule

Enzymes capable of activating the carboxyl group of certain amino acids have been ob tained from bacteria and other micro organ isms, as well as from animal and plant tissues Activation, at least in one form, results when an amino acid ATP and an appropriate enzyme interact to produce a mixed acid an hydride of the amino acid and adenosine monophosphate (with elimination of pyro phosphate) Activated amino acid residues then appear to be transferred to this or that template where they may be presumed to exist transiently as carboxyl activated derivatives Polypeptide formation would then come about

* See Lipmann 1956 Hoagland et al 1956 DeMoss and Novelli 1955

TERMINAL PORTION OF A POLYPEPTIDE CHAIN



through the orderly reaction of the activated carboxyl group of each amino acid residue with the amino group of the immediately adjacent residue It must be pointed out that the above picture of polypeptide formation is inferential in various respects and that some investigators hold that the synthesis of protein polypeptides may involve the participation of oligopeptides as precursors A portion of a polypeptide chain is shown here

In the last two phases of protein synthesis newly formed polypeptide chains are thought to dissociate from their respective templates and to undergo final shaping The latter process may be more or less complex and may involve for instance the aggregation of polypeptide chains and their folding to give the finished 3 dimensional protein

Small Molecule Control of Protein (Enzyme) Synthesis The last two phases namely the separation of polypeptide chains from templates and the final shaping of proteins have been implicated in certain control mechanisms that can regulate the production of some enzyme proteins One such mechanism enzyme induction (or enzymatic adaptation see reviews by Monod and Cohn 1952 Spiegelman and Campbell 1956) permits an enhanced synthesis of certain enzymes in response to specific small molecules such as the *substrates* (or chemically related substances) of the respective enzymes (see also p 62) Enzyme induction has been observed in microorganisms including bacteria as well as in animal and plant tissues For example some bacteria that under various cultural conditions produce relatively low levels of an enzyme capable of hydrolyzing the sugar lactose will synthesize much larger amounts of the enzyme if cultivated in the presence of lactose The enzyme thus synthesized brings about the cleavage of the lactose into its component monosaccharides which become available to the metabolic machinery of the organisms While enzyme induction is thus seen to be a valuable regulatory device it is possible under certain conditions to induce the formation of enzymes that are of no apparent advantage to the cells involved

Another regulatory device that can affect enzyme formation is enzyme repression (Vogel 1957) This device can bring about a decrease in the rate of synthesis of certain enzymes in response to specific small molecules such as the end products (or chemically related substances) of the biosynthetic pathways in which the respective enzymes participate When for instance one of certain amino acids is added to susceptible bacterial cultures there will result a lowering of the levels of one or more enzymes concerned with the *endogenous* formation of this amino acid (with concomitant utilization of the *exogenous* supply of this amino acid) It would appear then that enzyme induction and enzyme repression are complementary control mechanisms In either case the cell tends to form enzymes when they are needed and tends not to form enzymes when they are not needed

Nucleic Acid Synthesis * While enzyme induction and repression undoubtedly play important roles in the physiology of bacteria and other cells perhaps the most striking directive influence in protein biogenesis is that thought to be exerted by the templates The intriguing question of the chemical nature of templates has not yet been answered conclusively However there are good grounds for the belief that the nucleic acids (see p 67) contain within them the information † (i e the patternization code for protein synthesis) that is expected of templates

The nucleic acids are macromolecules whose small molecule building blocks are the nucleotides There are two major types of nucleic acids deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) depending on the sugar moiety (deoxyribose or ribose) of the respective component nucleotides The structures of some nucleotides and of a portion of a nucleic acid chain are shown here The way in which nucleic acids may store information for protein synthesis and a further discussion of nucleic acid structure can be found on p 67 Many investigators believe that such information can be contained in either DNA or RNA The possibility exists that DNA is a primary template from

* See reviews by Ochoa and Heppel 1957 Kornberg 1957

† The term information is used here and also on p 53 in reference to molecular structure the same term has also been employed in relation to hereditary determinants (see p 61)

unit (e g , a glucose residue) and a single type of bond between such units Polysaccharides of bacterial plant or animal origin, such as starches or dextrans are synthesized in an apparently step wise manner from suitable sugar derivatives as illustrated here Macro molecules of such a relatively low degree of distinctiveness are synthesized by enzymes much like those that mediate the synthesis of small molecules At the other end of the scale of distinctiveness are the proteins, including the enzymes which generally are composed of some 20 different amino acids linked in such a manner that each protein species comprises an apparently characteristic array of several dozen hundreds or even many thousands of individual amino acids

PROTEIN SYNTHESIS * As noted earlier (see p 40) the specific arrays of structural units present in proteins presumably cannot be pro duced through the agency of enzymes such as those that catalyze the formation of small molecules Rather the biogenesis of proteins is thought to depend on multifunctional cata lysts termed templates

Templates appear to be concerned with the emergence of the characteristic amino acid sequence (patternization) of the polypep tide chains Patternization however is not the only event in protein biogenesis The entire process involved may be viewed as consisting of the following phases

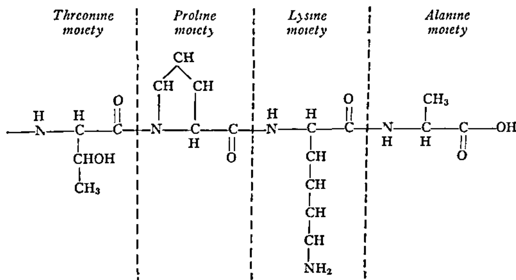
1 Biosynthesis (or uptake) of amino acids
2 "Activation' of amino acids
3 Transfer of activated amino acids to templates
4 Polypeptide formation from active pat ternized amino acids
5 Separation of polypeptide chains from templates
6 Final shaping of proteins

The first two phases deal with small molecule precursors of protein the next two phases with the synthesis of the polypeptide chain and the last two phases with the pro duction of the 'finished' protein molecule

Enzymes capable of activating the carboxyl group of certain amino acids have been ob tained from bacteria and other micro organ isms as well as from animal and plant tissues Activation at least in one form results when an amino acid ATP, and an appropriate enzyme interact to produce a mixed acid an hydride of the amino acid and adenosine monophosphate (with elimination of pyro phosphate) Activated amino acid residues then appear to be transferred to this or that template where they may be presumed to exist transiently as carboxyl activated derivatives Polypeptide formation would then come about

* See Lipmann 1956 Hoagland et al 1956 DeMoss and Novelli 1955

TERMINAL PORTION OF A POLYPEPTIDE CHAIN



which RNA is derivable as a secondary template

As far as the synthesis of nucleic acids is concerned some highly suggestive evidence is available Enzymes are known which will catalyze the formation of RNA like molecules from nucleoside diphosphates (such as ADP) and of DNA like molecules from deoxy nucleoside triphosphates (such as the deoxy analogue of ATP) However details as to the transfer of information in the course of nucleic acid synthesis remain to be elucidated

Bacteria as Atypical Cells

Bacteria as a Distinctive Group* In the foregoing discussion numerous similarities between bacteria and other types of cells have been considered Let us now examine certain features that distinguish bacteria from other cells Such features would seem to be of interest from the medical point of view in at least two respects In the first place the identification of bacteria partly rests of course on the recognition of traits that differentiate bacteria from other cells particularly other microbial cells Secondly an understanding of physiologic mechanisms that are peculiarly bacterial would be desirable in connection with rational approaches to chemotherapy since earches for chemotherapeutic agents could then be directed toward the discovery of substances that would interfere selectively with bacterial metabolism (and not with host metabolism) While it is hardly possible to point to any particular attribute *uniquely* characteri tic of bacterial cells there are features that are more or less regularly associated either with the bacteria as a group or with certain of their major subgroups

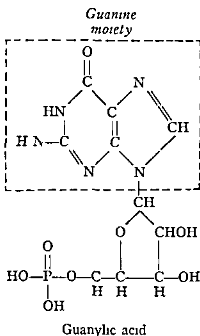
It has long been known that the bacteria and a related group of organisms the blue-green algae differ from other cells in exhibiting no microscopically visible cytoplasmic streaming or vacuoles More recently another trait has been discovered which again indicates that the bacteria and the blue green algae are set apart from the rest of the biologic world the occurrence of the 7 carbon amino acid α ε diaminopimelic acid (DAP see p 54) in one or another isomeric form appears to be largely confined to the bacteria and the blue-green algae (see review by Work 1955) DAP was found in all of numerous bacteria and blue green algae examined with the exception of a group of gram positive bacteria which include certain cocci and lactobacilli in contrast, DAP could generally not be detected in fungi nonblue green algae protozoa or higher forms of life In some sense then the gram positive bacteria contain transitional types and the gram negative bacteria (*all* of which appear to contain DAP) are even more remote from the higher forms than are the gram positive bacteria The unique taxonomic and evolutionary position of the gram negative bacteria (and blue green algae) is further illustrated by the distribution pattern of the enzyme ornithine δ transaminase which participates in amino acid metabolism ornithine δ transaminase activity has been detected in higher animal and plant tissues in protozoa nonblue green algae fungi and all of the gram positive bacteria investigated but *not* in various kinds of gram negative bacterial and blue green algal cells (Scher and Vogel, 1957)

Some Special Physiologic Mechanisms Among Bacteria The above mentioned several differences between bacteria and other organisms either represent or imply special mechanisms in bacterial physiology However one is impressed not only with these and other dissimilarities between bacteria and other forms but also with the extensive differences in physiologic mechani ms that exist *among the bacteria themselves*

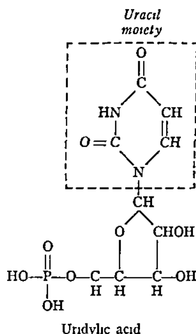
This physiologic diversity among the bacteria is pointedly reflected in the broad range of environments that will support bacterial growth For instance some bacteria will grow in media containing substantial concentrations of free mineral acids (at pH < 1) others will thrive in the presence of free ammonia We also find wide variations in sensitivity to or dependence on such environmental conditions as salt concentration and hydrostatic pressure additional illustrations of such diversity can be encountered in many other aspects of bacterial nutrition and cultivation Instances of high specialization as well as cases of great versatility can be observed For example certain

* This section is primarily concerned with *physiologic* distinctiveness for *morphologically* distinctive bacterial f atu es ncluding cell walls and flagella see pp 3 and 35

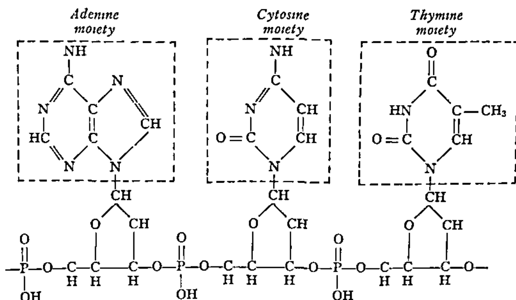
TWO MONONUCLEOTIDES (RIBONUCLEOTIDES) AND A PORTION OF A DEOXYRIBONUCLEIC ACID CHAIN



Guanylic acid



Uridylic acid

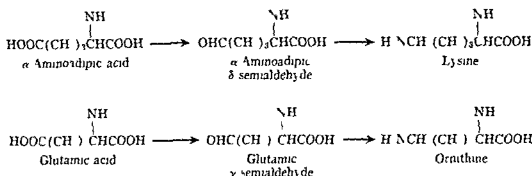


Portion of a deoxyribonucleic acid

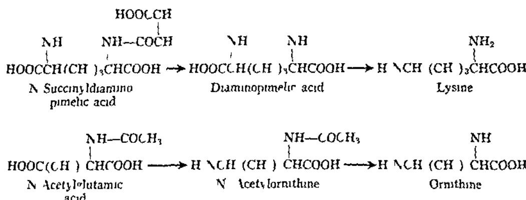
Guanylic and uridylic acids as well as adenylic and cytidylic acids can be obtained as degradation products of ribonucleic acids These four mononucleotides correspond to the bases guanine uracil adenine and cytosine respectively Guanine and adenine are purines, uracil and cytosine are pyrimidines Deoxyribonucleic acids generally contain guanine adenine cytosine and thymine (but no uracil) moieties Thymine is a pyrimidine

PATHWAYS OF LYSINE AND ORNITHINE FORMATION IN THE FUNGUS NEUROSPORA CRASSA AND IN THE GRAM-NEGATIVE BACTERIUM ESCHERICHIA COLI

Neurospora crassa



Escherichia coli



In N. crassa *the homologous amino acids lysine and ornithine are synthesized from homologous ω semialdehydes. In* E. coli *the two amino acids are produced via N-acylated intermediates; the acyl groups involved are succinyl and acetyl for lysine and ornithine respectively.*

thetic mechanisms that yield substances of more limited distribution than that of DAP, lysine and ornithine. Among such substances are a variety of small molecules including pigments and antibiotics, and an assortment of distinctive macromolecules such as those associated with surface structures, as well as storage materials and toxins.

Comments on Bacteria as Atypical Cells. At this juncture the reader may feel that after the relatively detailed presentation of the *similarities* between bacteria and other forms of life, the section on bacteria as *atypical* cells does not do justice to the fact that there are after all tremendous and perfectly obvious differences between a bacterium and, say, a higher animal. It must be remembered, however, that we have been concerned with comparisons of bacterial *cells* with *cells* of other forms. As far as we can judge from presently available evidence (which is primarily concerned with qualitative metabolic patterns), the impression seems to be inescapable that cells in general—bacterial and others—indeed have much in common from the biochemical point of view. The differences between unicellular bacteria and multicellular animals as

TABLE 1 SOME FERMENTATION END PRODUCTS DERIVABLE FROM PYRUVATE

TYPE OF FERMENTATION	BACTERIUM	CHARACTERISTIC PRODUCTS
Alcoholic	*Zymomonas lindneri**	Ethanol carbon dioxide
Homofermentative lactic	*Streptococcus lactis*†	Lactic acid
Heterofermentative lactic	*Leuconostoc mesenteroides*	Lactic acid ethanol carbon dioxide
Mixed acid	*Escherichia coli*	Lactic acetic formic and succinic acids ethanol hydrogen carbon dioxide
Butylene glycol	*Aerobacter aerogenes*	Same as from mixed acid fermentation plus 2 3 butylene glycol and acetoin
Butyric acid butanol acetone	*Clostridium acetobutylicum*	Butyric and acetic acids butanol ethanol acetone
Propionic	*Propionibacterium arabinosum*	Propionic and acetic acids carbon dioxide

* In this organism glucose yields pyruvate via a special pathway (rather than via the glycolytic pathway)
† Compare p 43

bacteria utilize (sometimes obligatorily) very special sources of carbon in contrast other bacteria can derive their carbon entirely from any one of scores of different organic compounds Interesting types of physiologic specialization in bacteria can also be seen in unusual combinations of energy sources and oxidizable substrates utilized Thus whereas most organotrophs (see p 40) derive their energy from *chemical* reactions *certain organotrophs depend on radiant* energy and use organic substances as hydrogen donors for the reduction of carbon dioxide in photosynthesis (*photo organotrophy*) Another type of physiologic specialization (*chemolithotrophy*) characterizes those lithotrophs (see p 40) which instead of radiant energy utilize energy from oxidation reduction reactions (involving inorganic oxidizable substrates such as thiosulfate)

SOME SPECIAL FERMENTATIONS * The physiologic diversity among the bacteria is illustrated further by their manifold fermentative activities which frequently are highly characteristic and of taxonomic value Numerous types of compounds will serve as substrates for bacterial fermentations and a given substrate may be fermented to a number of different end products *For example some* end products derivable from pyruvate (a key intermediate in the fermentation of glucose see p 43) are listed in Table 1 Fermentation is by no means restricted to carbohydrates such as glucose but can also occur with other substrates such as amino acids and purines

SPECIAL BIOSYNTHETIC MECHANISMS We have already seen that most bacteria contain a special amino acid DAP, which is not *found in higher organisms In this connection* it is noteworthy that in at least some bacteria (e g *E coli*) DAP functions not only as a cell wall component (see p 33) but also as an intermediate in the formation of the amino acid lysine It is perhaps no accident that the path of lysine synthesis in *E coli* (via DAP) *differs strikingly from the path of lysine* synthesis in such organisms as yeasts and fungi (which do not contain demonstrable amounts of DAP) * Bacteria such as *E coli* also differ from yeasts and fungi in the synthesis of ornithine a lower homologue of lysine Interestingly enough ornithine and lysine are produced through one set of (at least partly) analogous reactions in *E coli* and through another in yeasts and fungi (see accompanying diagram and review by Vogel and Bonner 1958) The existence (in a bacterium on the one hand and in yeasts and fungi on the other) of different *pathways* leading to a given metabolite can be taken as yet another indication of the distinctive position of bacteria in evolution Moreover the existence of such different pathways is consistent with the view that the metabolites to which they lead (lysine or ornithine) are from the point of view of evolution more ancient than the pathways themselves (see the discussion by Horowitz 1945)

Some bacteria also have special biosyn

* See Thimann 1955 Barker, 1956

* Details of lysine biosynthesis will be found in Work 1955 Gilvarg 1957 see also Scher and Vogel 1957

ganic form, and glucose has already been mentioned as a substrate that is widely utilized by such organisms. In addition, one or more of a remarkable variety of organic substances can be used as exclusive or partial sources of carbon by different strains of bacteria. These substances include numerous alcohols, fatty acids, amino acids, carbohydrates, and many other organic compounds of greater or lesser complexity. In some cases, notably in the genus *Pseudomonas*, we find individual strains that can utilize as sole carbon source any one of a relatively large number of organic substances.

Among the sources of *nitrogen*, ammonium salts or nitrates are used by many bacteria. However, some bacteria require substantial quantities of *organic* nitrogen-containing compounds. Certain other bacteria are able to satisfy their nitrogen requirement by fixing molecular nitrogen from the air.

The element *sulfur* can also be provided or may be required in one of several chemical form, including sulfate, sulfite, sulfide, thiosulfate, molecular sulfur, and others. Some organisms need a source of organically bound sulfur, e.g., cysteine.

The elements *hydrogen* and *oxygen* may be derived from the water of the medium and, depending on the bacterium and growth conditions used, from certain organic compounds, from inorganic salts, or, respectively, from gaseous hydrogen and oxygen.*

Phosphorus and *molybdenum* are usually supplied as the phosphate and molybdate anions. The remaining elements, including *potassium*, *magnesium*, and the *trace elements* (other than molybdenum), are generally provided as cations.

GROWTH FACTORS. In addition to the nutritional requirements discussed above, many bacteria need, in relatively small amounts, one or more organic compounds other than their main carbon source. Such specific compounds, or *growth factors*, include vitamins, amino acids, purines, and pyrimidines. In general, adequate growth factor concentrations are in the range of a fraction of a milligram to 1 milligram of a vitamin per liter and a few milligrams to about 100 milligrams of an amino acid, a purine, or a pyrimidine per liter. Growth factors usually can be regarded as organic substances that the cell needs for its metabolic activities but is unable to synthesize from its main carbon source. Additionally, growth factors can, of course, serve as contributory sources of all those elements (e.g., carbon, nitrogen, sulfur) of which they are composed. Rather complex growth factor requirements are observed in some cases (see p. 58).

HYDROGEN ION CONCENTRATION, BUFFER ACTION, AND SALT CONCENTRATION. As mentioned above, a number of the required elements generally are supplied in ionic form, i.e., as salts. Certain salts can also have other functions in the cultivation of bacteria, namely, the provision of appropriate hydrogen ion concentrations as well as buffer action. For example, hydrogen ion concentrations in the region of neutrality (pH 7) can be furnished by suitable mixtures of monobasic and dibasic phosphate. The buffer action, i.e., the tendency toward maintenance of a particular pH, can be influenced by the total concentration of the phosphate mixture. In some cases, an undesirable lowering of pH (due to acid formation by bacteria) is conveniently prevented through the incorporation of solid calcium carbonate into the growth medium. This sparingly soluble carbonate, in the presence of acid, decomposes with the evolution of carbon dioxide and tends to maintain the pH of the medium near neutrality.

A third function of salts is concerned with *osmotic pressure*. Many bacteria tolerate relatively large changes in the salt concentration of the medium. However, some bacteria are more sensitive in this respect; among them are the halophilic organisms, which require comparatively substantial salt concentrations.

GROWTH MEDIA. Many specific media have been described that are especially suitable for the cultivation of particular organisms; however, frequently a given organism can be cultivated on a number of different media. The choice depends not only on the bacterial strain involved but also on the purpose for which the latter is grown. Some possible alternatives in the designing of a medium are (1) complex medium (of ill-defined chemical composition, e.g., one containing peptone or yeast extract) versus defined medium (i.e., one of known chemical composition); (2) selective

*In their response to atmospheric oxygen, bacteria range from *obligately aerobic* forms (which cannot grow without molecular oxygen) to *obligately anaerobic* ones (which cannot survive in the presence of even relatively minute amounts of molecular oxygen). *Facultatively anaerobic* bacteria can grow (under suitable cultural conditions) either in the presence or the absence of air. *Microaerophilic* organisms require molecular oxygen at partial pressures less than that of oxygen in air.

organisms may not be explainable solely in terms of metabolic patterns such as the ones so far analyzed An understanding of these differences presumably will require more detailed knowledge regarding (1) the nature of the interactions *among* cells in multicellular organisms (2) the nature of *intracellular* factors that support such interactions and that in turn probably are a function of (3) the manner in which the metabolic activities within individual cells are organized in relation to space and time

Nutrition and Cultivation of Bacteria*

Some of the salient features of bacterial nutrition have been mentioned briefly in earlier sections Thus it was pointed out that in general bacteria utilize small molecular nutrients which furnish both materials and energy for the needs of the cells and may be transported into the cells with the aid of more or less specific mechanisms The nature and the relative proportions of available nutrients will of course exert profound influences both general and specific on cellular metabolism

Some specific influences such as those of particular substrates have already been considered in connection with the control of enzyme formation (induction and repression) Another relevant control mechanism which appears to be concerned with enzyme *activity* (rather than enzyme formation) is reflected in the utilization of an *exogenously* provided substance in preference to the same substance produced *endogenously* For example when certain amino acids are added to susceptible growing bacterial cultures (in a medium which had been free of such amino acids) the added amino acids will be taken up rapidly and will be utilized (for protein formation) to the more or less complete exclusion of the corresponding endogenous amino acids Such preferential utilization of exogenous substances is valuable to cells since it curtails a temporarily unnecessary flow of metabolites through certain pathways Further economy can result from a *coaction* of preferential utilization and of enzyme repression in such cases the biosynthesis of both metabolites and enzymes participating in the pathways involved tends to be reduced This elimination of superfluous biosynthetic operations coupled with the availability of appropriate nutrients presumably contributes to the more rapid and extensive growth of many bacteria on *complex* media compared with the growth obtained on so called minimal media

* See Stanier et al 1957 Cf pp 40 53 54

Elements Required for Growth As mentioned before certain elements are generally required for the nutrition of bacteria (and other organisms) These elements may be divided into two groups namely the non metallic ones and the metallic ones The non metallic ones (carbon, hydrogen oxygen nitrogen sulfur and phosphorus) as we have seen function as structural components in the organic (carbon containing) substances of the cell The metallic elements (potassium magnesium calcium iron manganese cobalt copper zinc and molybdenum) which usually are supplied in ionic form are essential by virtue of their participation in enzyme catalyzed reactions and in at least some cases presumably have additional functions Certain of the metallic elements are required in such small amounts that they are known as *trace elements*

Frequently depending on the medium used bacteria are found to contain elements in addition to the above mentioned 15 essential ones For example when an organism such as *E coli* is cultivated in media containing sodium ions considerable quantities of these ions are demonstrable in the cells However *E coli* does not seem to have a requirement for sodium ions since it can be grown in the absence of sodium (except for possible traces unintentionally introduced with the chemically pure components of the medium cf p 58)

Provision of the Required Elements As noted earlier the elements required for growth must be provided in a suitable chemical form As illustrated in the following section both versatility and specialization with respect to such chemical form are encountered among the bacteria

Sources of Elements The main (and usually only) *carbon* source for lithotrophs is carbon dioxide (or a carbonate) * The chemo organotrophs of course require carbon in or

* Organisms that utilize carbon dioxide as their only carbon source are sometimes termed *autotrophs* (in contrast with *heterotrophs* which require organic compounds as nutrients)

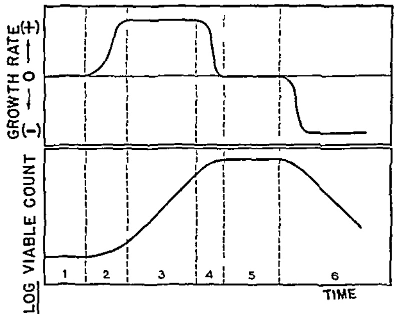


FIG 10 Phases of discontinuous bacterial growth (Lower curve) Log viable count (in arbitrary units) as a function of time (Upper curve) Corresponding changes in growth rate The vertical broken lines separate various phases (see text)

by available nutrients and by physical forces (e g 10^{13} bacteria per ml of liquid would yield an almost solid mass) but also by restrictions on continued multiplication that occur as a result of environmental changes produced by the biochemical activities of growing bacteria Only under exceptional experimental conditions involving the constant supplementation of bacterial cultures with fresh nutrients and the simultaneous removal of inhibitory metabolites and of surplus numbers of bacteria (see p 77) can constant growth rates be maintained for prolonged *periods of time Under the more frequently* encountered condition of bacterial multiplication namely within a closed environment such as a culture tube gradual environmental changes can occur which eventually have significant effects upon bacterial multiplication These effects are reflected in the production of a typical growth cycle which can be divided into a number of separate phases

Before discussing such growth cycles a few remarks must be made concerning the manner in which the number of bacteria in a population can be measured The *total number* of bacteria can be enumerated by direct microscopic counts on culture samples or can be approximated by turbidity measurements The *viable count* representing the *number of bacteria capable of reproduction* can be obtained by plating appropriately diluted and dispersed samples on a suitable solid medium and counting the number of colonies that develop upon incubation It is this viable count that will primarily concern us in the following discussion of definable phases of growth of a typical bacterial culture

As illustrated in region 1 of Figure 10 bacteria that have been removed from an aged culture and have been inoculated into a fresh growth medium will fail to multiply for a period of time This initial period during which the growth rate is zero is referred to as the lag period or lag phase The period labeled 2 in Figure 10 (acceleration phase) is the phase of initiation and gradual acceleration *of cellular multiplication* and hence of cultural growth During the next phase (3) a constant rate of reproduction is achieved leading to an exponential increase in population density with respect to time If in this phase (exponential phase) the logarithm of the viable count is plotted as a function of time (Fig 10) a linear relationship is obtained (For this reason the exponential phase is sometimes referred to as the logarithmic phase) Increases in both viable and total cell counts are identical during this phase However as already noted above the

medium (e g , a nitrogen free one which would select for nitrogen fixing organisms) versus nonselective medium (e g , so called nutrient broth which permits the cultivation of many different bacteria) (3) liquid medium (which is particularly suitable for mass culture) versus solid medium (which is solidified with 1 5% agar and supports colonial growth) (4) media that will allow rapid and extensive growth (say for the purpose of a high yield of bacteria) versus media that will yield particularly hardy vegetative cells or spores (for storage as stock cultures) and (5) media adapted to continuous cultivation (in which certain nutrients may be provided at unusually low concentrations see p 77) versus media for the usual (discontinuous) cultivation

As an illustration let us consider a simple medium that will support the growth of many chemoorganotrophic bacteria It must contain the 6 essential nonmetallic elements plus potassium and magnesium adequate amounts of the trace elements are often present as impurities in the other ingredients even when the latter are used as the chemically pure commercial products An example of such a medium would be one containing water glucose ammonium sulfate magnesium sulfate and a suitable mixture of monobasic and dibasic potassium phosphate In contrast with such a simple medium suitable for the growth of many bacteria is a lactobacillus medium which must contain several dozens of amino acids vitamins and other compounds as growth factors

It is often desirable to supplement media with a compound such as citrate which may have beneficial effects as a solubilizing and chelating agent Chelation may overcome the toxicity of certain heavy metals for some cells Another valuable detoxification procedure is the incorporation of serum albumin into media for the cultivation of *Mycobacterium tuberculosis* the success of this method depends on the ability of albumin to adsorb toxic fatty acids (Dubos and Davis 1946) In the case of anaerobic bacteria the toxic effects of molecular oxygen can often be counteracted by the addition of reducing agents (such as thioglycolate or cysteine) to the culture medium

THE ATMOSPHERE AS A FACTOR IN NUTRITION The role of atmospheric oxygen in the case of aerobic bacteria and that of nitrogen in the case of nitrogen fixing bacteria have been discussed earlier In addition the atmosphere can function as a source of carbon dioxide which may be required even by organotrophic bacteria especially in the initiation of growth For certain purposes atmospheric conditions can be modified experimentally for example the atmosphere may be excluded altogether (e g , in the cultivation of anaerobic bacteria), or gaseous hydrogen may be provided (e g , in the cultivation of hydrogen utilizing bacteria) Under laboratory conditions, it is sometimes desirable to enhance the extent of contact between bacteria in a liquid culture and the surrounding atmosphere, such enhanced contact can be achieved for example by continuous or intermittent agitation of the culture Of course many different kinds of gaseous environment can be found in the natural habitats of bacteria

Physical Factors Relating to Nutrition* An important physical factor in the cultivation of bacteria is *temperature* which in the laboratory is controlled through the use of constant temperature incubators, water baths or the like Some bacteria (*psychrophils*) grow best at temperatures below 20° C some (*mesophils*) between 20 and 45° C and some (*thermophils*) between 45 and 80 C Many bacteria are able to *survive* exposure to temperatures considerably above the maximum or below the minimum temperature at which growth can still occur In the case of photosynthetic bacteria of course *illumination* must be provided The commonly encountered bacteria are rather insensitive to moderate *hydrostatic pressure* however relatively elevated pressures can be injurious In contrast deep sea bacteria frequently cannot grow unless they are put under high pressures comparable with those of their natural habitat

GROWTH OF BACTERIAL POPULATIONS†

Bacterial multiplication usually proceeds by binary fission hence the population size will increase in an exponential fashion, one bacterium giving rise to 2, these in turn to 4, then 8 etc The average time that elapses between two successive divisions is the mean generation time and the growth rate is the reciprocal of that value If there were no limit to the size of a bacterial population and if the environmental conditions remained constant bacteria could be expected to propagate indefinitely at constant growth rates However bacterial population sizes are limited not only

* See Porter 1946

† Reviewed by Monod 1949

will be able to grow and hence will present the appearance of an unusually long lag period of the whole culture

Presumably a lag period is not only an in vitro phenomenon but also occurs when pathogenic bacteria invade an animal host Under the latter conditions the duration of the in vivo lag phase may have a significant influence on the outcome of host parasite interactions since the duration of the lag phase represents the time during which natural host defenses can act on the initially often quite small number of nonpropagating parasites A presumably important factor determining the actual duration of the in vivo lag is the physiologic state of the invading organisms Since this state is a function of the bacteria s prior environment and growth phase the potential influence of the prior history of the parasite population upon the course of infective processes becomes quite apparent

The rate of bacterial growth during the exponential phase is a more or less distinct characteristic of each strain When organisms are growing under apparently optimal conditions the average generation time during this phase can be as low as 15 minutes in some species more generally it is of the order of 30 to 60 minutes and in the case of some organisms e g tubercle bacilli may be as long as 15 hours or more Naturally the duration of such generation times can be affected materially by changes in environmental conditions such as alterations in the concentration of nutrients shifts to suboptimal pH and temperature ranges etc

Intimately connected with considerations of generation time is the question of the onset of division of individual bacteria in a culture In the exponential phase a growing culture as a rule will contain some cells that have just divided others that are about to divide and still others that are in intermediate stages However in recent years a number of methods have been developed which permit the synchronization of cellular divisions in bacterial cultures Most of them are based on the fact that conditions which temporarily inhibit cell division will permit individual cells to progress in their growth up to a point just prior to their division then when normal growth conditions are reinstated all cells will undergo division simultaneously and will maintain their synchronization for a number of successive divisions Temporary chilling of bacterial populations has proved to be particularly effective for the production of such synchronized growth another method has utilized the reinitiation of DNA synthesis in thymine requiring mutants which previously had been deprived of thymine (Barner and Cohen 1956)

Finally a variety of conditions can be responsible for the eventual cessation of growth in all bacterial cultures maintained in a closed system The accumulation of toxic metabolic products unfavorable changes in pH the gradual reduction in availability of oxygen in liquid media in the case of aerobically growing bacteria are predominant causes The depletion of required nutrients is a far less frequent cause for the attainment of the stationary phase and for the subsequent death of bacteria

BACTERIAL GENETICS*

Sources of Variability

Both morphologic and physiologic characteristics of bacteria are subject to considerable variability One of the significant advances in the field of bacteriology during the last two decades has been the demonstration that the factors influencing such variability are in many respects comparable with those affecting higher organisms As in the latter it is important to distinguish two broad classes of variation namely changes affecting the *genotype* (sum total of hereditary determinants) and changes affecting the *phenotype* of organism Genotypic changes involve relatively stable alterations of the determinants of hereditary characteristics which are carried by and transmitted from cell to cell primarily via nuclear components Phenotypic changes merely modify the actual expression or manifestation of the traits which are under the control of the genotype Thus each cell transfers to its daughter cell a set of determinants also often referred to as *information* which determines the cell s ability to react in a specific way to specific environmental conditions

* More detailed discussions may be found in Braun 1953 McElroy and Glass 1957

duration of this period is usually limited to a few hours or at most, to a few days since, as a rule the attainment of high population density is accompanied by rapid changes in the environment including the exhaustion of nutrients and the accumulation of toxic products As a result growth rates begin to decline (phase 4 in Fig 10) Eventually, increases in viable number of bacteria will cease and the viable count will reach a constant level During this so called stationary phase (5) the total number of bacteria also may remain constant (e g in the case of total exhaustion of nutrients) or the total number may continue to increase while the viable number remains constant (The latter condition can produce an intense struggle for survival since now only a limited number of bacteria that arise will retain the capacity for reproduction Thus this phase frequently becomes of great significance for population changes which may occur due to the selective multiplication of fitter mutants see p 76) Unless transferred to fresh media the members of a bacterial population eventually will die Such death during the ' phase of decline or death phase (6) expresses itself in a gradual decrease of the viable count Depending upon the species death may be followed quickly or slowly by a decomposition or lysis of the dead cell lysis expresses itself in a decrease of the total count

The above mentioned changes in rate of multiplication during the growth cycle of bacterial populations are generally associated with some changes in cellular morphology and in the average number of nuclei per cell Initially cells transferred from aged cultures to fresh media are often small and then grow larger during the acceleration phase During the exponential phase cells tend to be large whereas their progeny in the stationary phase tend to be smaller and less homogeneous in size In young cultures nuclear division may proceed slightly faster than cellular division thus giving rise to a preponderance of multinuclear cells Eventually the rate of cellular division catches up with that of nuclear division therefore cells in older cultures tend to be uninuclear Many physiologic properties of the cells also may change as a function of the phase of growth Thus some inhibitors e g penicillin, affect only actively growing cells, whereas others, such as ultraviolet irradiation, are said to affect resting cells more than they do growing cells

The actual duration of the various phases of a typical growth cycle of a bacterial population is not only dependent upon the genetic constitution of the cells used as inoculum but also upon a wide variety of environmental conditions that affect the physiologic properties of the cells Thus, the duration of the lag phase is attributable in part to the time required to synthesize intermediates and enzymes that are required for multiplication but had been exhausted during the cells prior residence in an old medium This replenishment effect is illustrated by the observation that inocula prepared from cultures in the exponential phase of growth exhibit a partial or complete abolition of the lag phase Frequently however even transfers of actively growing cells to fresh media may result in the occurrence of a lag period This may be ascribed to a variety of causes for example an initial inadequacy of fresh media to support growth Such initial inadequacy may be overcome subsequently as the result of the metabolic activities of the nondividing cells For example the CO_2 content of a fresh medium may be below that required for the growth of certain bacteria however the necessary concentration of CO_2 may be produced through the metabolic activities of the organisms during the lag phase and thus permit a delayed initiation of growth Frequently the lag period is associated with the time required for the formation of adaptive enzymes (p 62) which may be required for growth in the presence of a new substrate The formation of adaptive enzymes requires contact between the cells and the new substrate and is a time consuming process even when the inoculum is taken from an exponentially growing culture Finally in some cases of transfers of bacteria from one medium to another a phenomenon superficially resembling a prolonged lag phase may ensue due to the fact that the vast majority of the bacteria in the inoculum actually are unable to grow in the new medium only the progeny of a rare variant (mutant) cell present in the inoculum (or arising during limited residual growth of the inoculated type)

(see p 51) In these cases cells endowed with the genotypic potential to form specific enzymes will tend not to do so unless the corresponding substrate (or a chemical relative) which the enzyme is capable of attacking is present in the environment The specific substrate thus acts as an inducer of the enzyme in a genotypically competent cell One example of induced enzyme formation has been presented already in a prior section (p 51) Another example is provided by certain strains (e g of *Bacillus cereus*) which following contact with penicillin will produce greatly enhanced levels of the enzyme penicillinase (Pollock 1953) The potential capability for the induction of this enzyme is a heritable property of these organisms but the realization of this capability requires the proper environmental stimulus namely contact with penicillin In most instances the inducer of the enzyme is its substrate but in some more recently analyzed cases it was found that the inducer does not have to be the substrate but can be a chemically related substance Also in most cases induced enzyme formation ceases with the removal of the particular substrate yet in a number of adaptive enzyme systems for example penicillinase formation the enzyme can be formed for a considerable number of generations following removal of exogenous substrate Since in addition to the inducer the presence of the proper genotype is a prerequisite for all cases of enzyme induction a change in the relevant part of the genotype will lead to a loss of inducibility even in the presence of the inducer

Genotypic Variation

All of the above changes in the expression of physiologic and morphologic characteristics of bacteria can occur only within the limits of the reaction range determined by the cell s genotype It will be remembered that the genotype represents the sum total of hereditary determinants (genes) and that (in an extremely simplified way of speaking) this total is made up of one or more determinants for each heritable trait It is obvious that the limitations imposed by a fixed genotype could prove to be fatal to a living organism if it were suddenly confronted with an environment with which the cell cannot cope within the limits of its reaction range or in which it would be at a decided disadvantage in competition with other fitter microbes Actually such limitations can be overcome to a considerable though not full extent by the occurrence of occasional genotypic changes which can endow individual cells with an altered reaction range As in higher organisms there are two basic mechanisms in bacteria that can lead to genotypic changes namely mutations and genetic transfers between cells (sexlike processes)

Mutation The first mechanism mutation adds abolishes or modifies specific determinants (or information) These changes in individual determinants are generally considered to be associated with alterations of the DNA of intranuclear chromosomelike structures (see p 36) and occur spontaneously i e independently of specific environmental conditions Mutational events give rise to *mutant cells* or *mutants* The rate of mutation varies with the determinant (gene) involved and may be as low as 1×10^{-10} (i e 1 cell per 10^{10}) or as high as 1×10^{-4} per bacterium per generation or even higher Thus the rate of mutation from streptomycin sensitivity to resistance in *E coli* has been determined as 1×10^{-10} and mutations affecting the pigment production by *Serratia marcescens* have been found to occur at a rate of 1×10^{-4} In addition to resistance and pigmentation characteristics mutations affecting cellular and colonial morphology antigenicity virulence and nutritional requirements have been studied widely and will be discussed in more detail below Certain environmental agents such as ionizing radiations ultraviolet rays hydrogen peroxide carcinogenic substances nitrogen mustards and even simple salts like $MnCl_2$ are capable of increasing the rate of mutation significantly above the spontaneous rate The term *mutagens* has been applied to these agents At one time it was believed that such mutagenic agents have a very generalized effect increasing the rate of mutation of *all* genes above those occurring spontaneously under what we consider as normal environmental conditions However more recent investigations have revealed that specific genes tend to show individual responses to mutagenic agents, thus certain genes will display greatly increased mutation

in other words the genotype controls the cell s reaction range The environment in turn, influences the particular expression of a heritable trait or its phenotype, and thus can change or modify cellular processes temporarily with out any alteration of the genotype For example the genotype of most *Salmonella* cells endows them with the capacity to develop flagella However when such cells are maintained on media containing phenol flagella fail to develop This represents a phenotypic change or temporary modification since immediately after return to phenol free media the daughter cells or *progeny* will again develop flagella Another example of purely phenotypic alterations is the production of capsules by smooth anthrax bacilli maintained in a CO containing atmosphere and a lack of such encapsulation in genotypically identical cells growing in air These two examples may suffice to indicate that the interactions between the transmitted genotype (or information) and environmental influences can cause many divergent phenotypes despite the presence of identical genotypes This potential plasticity of expression within a given genotypic reaction range coupled with the constant though rare occurrence of changes in the genotype itself contributes to an exceedingly high degree of *detectable* variability in organisms like bacteria which propagate with unusual rapidity attain extremely large population sizes and are considered, as a rule to be haploid * There was a period in the not too distant history of bacteriology when much confusion and mysticism arose in connection with bacterial variability primarily due to a lack of clear distinction between genotypic and phenotypic changes a lack of recognition of factors that controlled the occurrence of such changes and a lack of understanding of factors influencing the establishment of genotypically altered cells in bacterial populations (population changes)

Phenotypic Variation

As pointed out above, even though environmental influences are capable of modifying the phenotype as a rule such modifications persist only in the presence of the specific environmental condition and will not be inherited by the offspring It is characteristic of such phenotypic changes to affect almost simultaneously all members of a bacterial population consisting of cells of comparable genotyp and to disappear almost immediately following the return of the population to prior environmental conditions Thus the interference by penicillin with the synthesis of certain cell wall components temporarily converts all so called smooth cells of certain bacterial populations maintained in subinhibitory concentrations of penicillin into what appear to be nonsmooth cells (lacking lipoprotein surface components) however all these cell immediately regain their smooth characteristics upon subcultivation in penicillin free media The prior contact with penicillin will in no way modify the information that such cells transmit to their progeny However it should be noted that a phenotype similar to the example just cited also can be produced occasionally by a spontaneous genotypi change which interferes with the cell s ability to synthesize the lipoprotein involved In th latter case only approximately 1 out of 10^7 cells (depending on the strain and the species involved) will undergo such a genotypic change (mutation) with a concomitant phenotypic change it is also important to note that such genotypic change would occur quite independently of the environment (see p 69) i e independent of the presence or the absence of penicillin in the above example Phenotypic alterations can affect temporarily the expression of practically any one of a multitude of heritable characteristics of bacteria often by altering biosynthetic reactions The very same biosynthetic steps that are temporarily altered by environmental changes frequently can also be altered more permanently by genotypic changes as already stated such events are independent of a specific environmental condition and in contrast with phenotypic alterations, are likely to affect at any given time only one or at the most a few cells in a population

A particularly well analyzed group of phenotypic modifications concerns *induced enzyme synthesis* or *adaptive enzyme formation*

* In a haploid organism each genetic determinant is present singly and not doubly as is the case in the generally diploid (or polyploid) somatic cells of higher organisms

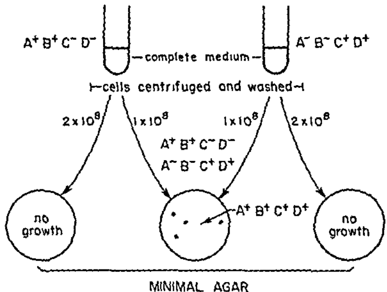


FIG 12 Occurrence of nutritionally independent recombinants (capable of growing on minimal media) following mixed cultivation of two strains of *E coli* possessing different growth factor requirements Note that neither parental strain will grow when plated on a minimal agar medium lacking growth factors A B C and D (A^- B^- etc denotes a requirement of the cells for growth factors A B etc respectively whereas A^+ B^+ etc denotes the absence of such nutritional requirements) (Braun W Bacterial Genetics Philadelphia Saunders)

1951) is also a relatively rare event in the competent bacterial strains in which it has been detected In transduction usually 1 sometimes 2 genes are transferred from a donor cell to the recipient cell (e g transfer of *d* to genotype *ABCDEFG* can yield *ABCdEFG*) The actual gene transfer occurs with the aid of bacteriophages (bacterial viruses)* which in the process of lysing a phage susceptible bacterium may pick up 1 or 2 bacterial genes and somewhat like a trolley car transport these into genetically different bacteria that are more resistant to lysis by phage In such host cells the phage establishes a rather permanent relationship known as *lysogeny* with the bacterium as a result of which the cell will continue to multiply normally and also will maintain and transmit to all its progeny the potentiality of producing phage of the same kind as the one that originated the infection In establishing such a lysogenic state the phage can introduce (transduce) specific genetic information unwittingly donated by the phage s prior bacterial host Such transductions have been detected primarily within and among many *Salmonella* species and more recently also between *Shigella flexneri* and *Escherichia coli* As in the case of recombination any hereditary characteristic controlled by nuclear genes (and this seems to cover the vast majority of them) appears to be transducable between competent strains

TRANSFORMATION At the lowest level in terms of the size of the transferred genetic material is the sexlike phenomenon that has been known to bacteriologists for more than 20 years namely bacterial transformation (see Austrian 1952 Ephrussi Taylor 1957 Hotchkiss 1957) This process first detected in pneumococci involves the incorporation of genetically active (naked) DNA derived from one bacterium into another bacterium belonging to a competent receptor strain Such transfers do not appear to depend upon any carrier (like phage) and as in the case of transduction usually are associated with the incorporation of a single new determinant into a recipient cell In so-called capsular transformations for example DNA isolated from encapsulated (S) type II pneumococci can cause a stable transformation of a small proportion of unencapsulated (R) cells into encapsulated (S) type II cells

The specificity of such transformations can be demonstrated in at least two ways (1) the

* See reviews by Lwoff 1953 Boyd 1956 Bertani 1958



FIG 11 Conjugation between cells of two highly interfertile substrains of *E coli* K 12 Giemsa stain after acid hydrolysis and osmic fixation (Lederberg J J Bact *71* 497 498)

rates following UV irradiation but little change from spontaneous mutability after exposure to Mn++ certain other genes have proved to react in the opposite way (Demerec 1955) Increased mutation rates for numerous genes can result not only from experimental environmental alterations but also from the action of specific genes (*mutator* genes Treffers et al 1954) that may arise spontaneously in certain strains

Sexlike Phenomena As is the case in higher organisms mutational events are not the only mechanism by which bacteria can undergo an alteration of their genotype The ability to combine within *one* organism different genetic changes which had occurred independently in two organisms obviously would provide a highly economic process for enlarging the potential reaction range and survival value of an organism Sexual processes in higher organisms accomplish such pooling reassorting or recombining of dissimilar genes existing among members of a species comparable processes have also been uncovered in a number of bacterial species In bacteria, however these processes differ in one important respect from those of higher forms in all hitherto detected sexlike processes of bacteria the genetic contributions made by the two parental cells are quite disproportionate In the sexual reproduction of higher forms both male and female germ cells contribute a comparable amount of genetic information however, where such processes have been found among bacteria one parental cell ("male") will contribute only a portion of its genotype which then may or may not be substituted for corresponding genes of the recipient cell ("female) To illustrate the general nature of such phenomena let us choose an example in which one parental cell (donor) contributes determinants (or "information" or "genes) represented by the symbols *abc* to the genotype *ABCDEFG* existing in the recipient cell (each letter in the preceding genotypic formula symbolizes a specific determinant e g *a* might represent streptomycin resistance *A* = streptomycin sensitivity *b* = inability to ferment lactose *B* = ability to ferment lactose etc) The resulting offspring then would have the genotype *abcDEFG* in which *abc* has been substituted for *ABC*

Three types of sexlike processes distinguishable by the size of the contribution made by the *donor* parent, have been recognized so far among bacteria

RECOMBINATION In so-called recombination (see Lederberg 1955) the two parental cells conjugate (Fig 11) and a sizable portion of the genotype of the donor cell containing a number of genes (as in the above example), is transferred to the receptor cell In the receptor cell all or part of the transferred genes may substitute for analogous genes previously present the latter (*ABC* above) are subsequently eliminated from the cell The actual transfer of the determinants presumably involves the cell to cell migration of a portion of nuclear material (probably comparable with a portion of a chromosome) However, such transfers are (with certain exceptions) relatively rare events In most strains in which recombination does occur and up to now this is restricted to certain strains of *E coli* it occurs among only a few members of such competent (fertile) populations e g, in 1 out of 10^5 cells The manner in which recombination between bacteria has become demonstrable is indicated in Figure 12

TRANSDUCTION The occurrence of another process of unidirectional genetic transfer, known as transduction (Zinder and Lederberg, 1952 Lederberg 1956b Hartman

ticular phage material (more precisely of prophage) in lysogenic cells (see p 65) can alter specific traits of the affected bacteria The production of toxin by lysogenic *Corynebacterium diphtheriae* and the manifestation of a new antigen in certain lysogenic *Salmonella* strains so far are the most prominent examples In the absence of the phage (which is detectable only by its ability to lyse phage sensitive indicator strains) toxigenicity of *C diphtheriae* or the antigen of *Salmonella* mentioned above are lacking Only infections by particular types of phage can cause these changes which therefore must be regarded as akin to genotypic alterations (and not as the results of lysogeny per se) It would appear then that a specific prophage takes over gene like functions in these cases The changes just described differ in their frequency from those previously discussed mutation recombination transduction or transformation of a given trait are relatively rare events likely to affect only a fraction of a given population In contrast the changes in bacterial traits produced by phage infections can affect every infected bacterium and thus can involve every member of a bacterial population

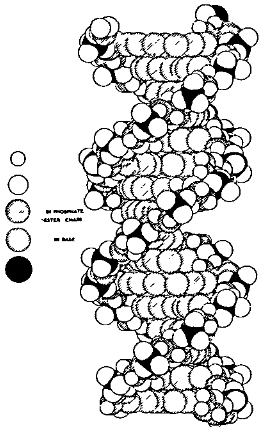


Fig 13 A proposed structure of deoxyribonucleic acid (L D Hamilton modified from Feughelman *et al* Nature 175 834 838)

Nature of the Hereditary Determinants*

Genetic and cytologic studies with higher organisms first indicated that the majority of determinants of hereditary traits are associated with nuclear elements specifically with chromosomes i e morphologic elements rich in DNA Studies with bacteria have confirmed the role of the nucleus as carrier of hereditary determinants even though the involvement of intranuclear chromosomal structures akin to those of higher forms has remained disputed Moreover the phenomenon of bacterial transformation mediated by highly polymerized DNA has supported prior assumptions that DNA contains genetic information However until relatively recently little information was available regarding possible DNA structures that would be in accord with existing physical and chemical data and would also be of sufficient complexity and allow for enough plasticity to account for the huge number of specificities associated with genetic determinants

In 1953 Watson and Crick suggested details of a DNA structure which meets many of the properties expected of a transmissible structure with manifold specificities They suggested a structure composed of two intertwined coils each of which consists of linked nucleotides The two nucleotide chains are held together by hydrogen bonds that pairwise connect bases one base of each pair belonging to one coil and the other base belonging to the other coil (Fig 13) The 4 bases that generally occur in deoxyribonucleotides are adenine (A) guanine (G) cytosine (C) and thymine (T) Watson and Crick proposed that the pairing of the bases is not random but follows a rule whereby adenine always occurs opposite thymine (A T) and guanine opposite cytosine (G C) In this manner any base

* Reviewed in McElroy and Glass 1957

mutation rate to S of most of the R strains that have been employed in these studies is so low that spontaneous R $\rightarrow$ S changes, as a rule do not occur (2) when R $\rightarrow$ S changes do occur spontaneously the S cells will display the same type specificity as the S cells from which the R type had originally been derived (e g S II $\rightarrow$ R $\rightarrow$ S II) yet in the presence of DNA from another encapsulated type (e g from S III) transformations will occur that establish the DNA donor's type specificity in the recipient cells (e g , S II $\rightarrow$ R $\xrightarrow{\text{DNA from S III}}$ S III) Correspondingly, DNA isolated from S type I cells can transform R cells derived from, for example S II into stable S type I pneumococci Such transformed S cells and their progeny in turn can yield DNA transforming R cells into S type I cells Many other characteristics have been transformed in the same way these include antibiotic resistance fermentative capabilities and morphologic characteristics

The transforming principle can be released from the donor cells by cell lysis or by extraction procedures designed to isolate DNA The critical role of DNA in these transfers of genetic properties is indicated by the fact that a brief exposure of the transforming principle to the depolymerizing enzyme deoxyribonuclease will abolish all transformation reactions This type of genetic transfer is not restricted to pneumococci but now has been shown to occur in several other bacterial species These include various species of the genus *Hemophilus* (where the occurrence of transformation between species also has been demonstrated) and also of the genera *Brucella Agrobacterium* and *Neisseria* The capability of DNA isolated from streptococci to transform pneumococci (in regard to antibiotic resistance) also has been demonstrated

Transformation is the only one of the sexlike processes in bacteria which is known with certainty to occur in vivo under experimental conditions In fact, the original demonstration of transformation dates back to the animal experiments of Griffith in 1928 which showed that virulent, living S pneumococci could be isolated from mice that succumbed following the combined injection of live avirulent R cells and heat killed heterologous S cells Thus S II $\rightarrow$ R $\xrightarrow[\text{+ heat killed S III}]{\text{into mice}}$ S III The natural occurrence of transformation phenomena is still unproved However, from the standpoint of infectious disease the potential significance of any sexlike processes in vivo involving genetic transfers between related strains of different virulence cannot be underestimated The possibility that such processes could lead to the occurrence of strains with unusual virulence is indicated by in vitro observations that unique transformations ("allogenic transformations) may occur in which the progeny displays heritable properties that differ both from those of the DNA donor and from those of the recipient cells Specifically transformations between two gentically dissimilar pneumococcal strains of intermediate virulence have given rise to transformants of high virulence Since virulence can be ascribed to a number of different heritable bacterial characteristics (e g resistance to phagocytosis, rate of reproduction in vivo toxin production etc), it is obvious that naturally occurring processes of genetic transfer between related strains of pathogens with dissimilar virulence could lead to high virulence due to the additive or synergistic effects of individual characteristics that contribute to virulence The potential significance of sexlike processes for naturally occurring alterations in virulence would not be diminished by the fact that the three mechanisms of genetic transfers in bacteria may occur only as rare events among a minute fraction of members of competent strains Since in susceptible hosts even an occasional bacterium of high virulence among a large population of less virulent relatives attains a high survival value the rare occurrence of a transduced transformed or recombinant cell with unusual virulence may suffice to produce a telling effect upon subsequent pathogenic and epidemiologic events

Lysogeny * Recent findings have indicated that to the above mentioned mechanisms of genotypic alteration in bacteria still another phenomenon must be added which like transduction is associated with bacteriophages It has been ascertained that the presence of par

* For references see footnote on p 65 and Luria 1953

'associated changes in cellular and colonial morphology as well as antigenicity p 74)

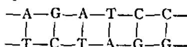
Demonstration of Occurrence of Mutations in Bacteria

The occurrence of spontaneous genetic changes *independent of specific environmental conditions* remained in dispute in the case of bacteria many years after acceptance of this fact in the case of higher organisms The known capability of bacteria to undergo heritable variation for example to become resistant to usually inhibitory conditions were at one time attributed principally to directed changes in response to environmental stimuli In contrast with this so called Lamarckian idea of inheritance of acquired characteristics stood the concept of mutation and selection The latter concept attributes such changes to the selective growth of mutants which arise spontaneously and independently of the environment and though occurring at low frequencies (e g 1 in 1 million) can rapidly outgrow their less fit parents under appropriate environmental conditions The reason for the long retention of Lamarckian ideas by earlier bacteriologists can be understood quite easily when it is remembered that in contrast with higher forms the rates of reproduction and the population sizes of bacteria are extremely high For example many bacteria have generation times as brief as 15 minutes and can rapidly attain average population sizes of 1 billion cells per ml Obviously under such conditions the fate of individual cells is obscured (unless special technics are employed) and changes in characteristics remain unrecognized until they affect many cells in the population In other words a change occurring in a single mutant cell will be recognized only after it has been transmitted to a sufficient number of descendants of the original mutant Since the progeny of a single cell is referred to as a clone one may say that ordinarily the occurrence of mutational events is recognized through detection of mutant clones rather than through the isolation of the original variant cell

To illustrate this further let us take as an example changes in nutritional requirements that may occur in a population of tryptophan requiring typhoid bacteria When a population of approximately 10^7 bacteria of this type is transferred into a broth medium free of tryptophan and cultural growth is determined by turbidity measurements only little, if any growth occurs initially However normal growth may occur in this supposedly deficient environment after some time and appropriate testing of the multiplying bacteria and their descendants will reveal that they actually are capable of growing without tryptophan When such growth was noted by earlier bacteriologists it was assumed to have been due to a slow adaptation of the original cells to a tryptophan free environment in their language the bacteria had been *trained* to grow in the environment More critical examination especially with the aid of viable counts subsequently demonstrated that actually the growth occurring in such cultures is not due to the capability of all or even of the majority of the members of the original population to initiate multiplication in the deficient environment Rather the growth is due to the selective multiplication of the descendants of one or a few mutant cells which arise with a frequency of approximately 1×10^{-7} and are capable of growing without tryptophan These mutant cells either had been present as a minority element in the inoculum or arose during the limited period of residual growth which cells with specific nutrient requirements may undergo even in a deficient medium at the expense of intracellular stored products

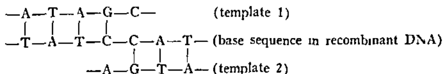
Comparable mutational events became easily demonstrable when selective media capable of supporting the growth of mutant cells and clones but not that of the parental cells were employed to detect with greater precision the nature of the changes that can occur in bacterial populations as the result of alterations in environmental conditions For example in the above cited case of change to tryptophan independence samples were removed from the broth culture and plated on tryptophan free agar medium where each tryptophan independent mutant cell could develop into a colony By counting the number of colonies that developed from samples taken at frequent intervals during the period of inapparent growth of the broth culture it could be shown that actually some multiplication

in one chain will predetermine its counterpart in the other chain and the sequence of bases in one helix or coil will determine a complementary sequence in the sister coil For example



Following separation of the coils each could serve automatically as a template that directs the assembly of nucleotides into a new helix containing the proper sequence of nucleotides It is precisely this kind of sequence that is believed to determine genetic specificity Despite the apparent involvement of only 4 different nucleotides (corresponding to the 4 bases) in the base sequences of DNA the number of possible arrangements is extremely high This can be visualized best by recognizing that words constructable from a 4 letter alphabet are not restricted to ABCD ACDB ADCB etc but also include BD ABCB CAA etc

As far as mutational events are concerned they presumably involve an alteration of the template in a restricted region Transformation or transduction may introduce new DNA templates for the synthesis of similar DNA from nucleotides in the recipient cell This possibility is consistent with the view that all cellular DNA synthesis proceeds by *de novo* assembly of nucleotide components to form highly polymerized DNA molecules Recombination in which the progeny displays characteristics that have been contributed partly by one parent and partly by the other might be regarded as the result of template skipping during DNA synthesis part of the new DNA would have been built up according to the template of the recipient cell and part according to the template supplied by the other parental cell For example



In the absence of further recombination or mutational events the new sequence and with it the new recombinant genotype would remain constant through subsequent generations This interpretation of the underlying mechanism is only one of several possibilities In fact, it should be recognized that the above picture of DNA replication though useful for the formation of working hypotheses, is speculative and may be greatly oversimplified Some investigators even suspect that the synthesis of new DNA may proceed in a fairly complex cycle involving both RNA and protein synthesis

Growing knowledge regarding the chemical structure of DNA combined with data that have been obtained from genetic experiments with micro organisms also have modified past concepts and definitions of the fundamental unit of heredity, the gene It is now recognized, for example that the smallest genetically active unit of DNA is not definable in simple terms The degree of resolution regarding the smallest genetically active region depends upon the method of analysis employed Thus the size of the smallest DNA region with specific activity recognizable by mutational events can differ considerably from that recognizable by recombination Regions of distinct genetic activity resolvable by recombination can be extremely small possibly involving only a few nucleotides (Benzer 1957 Demerec et al 1956)

Gene Action

Regardless of the size and the specific mode of replication of the carrier of genetic information the next question that arises is how do specific regions of the giant DNA molecule control the development of specific physiologic and morphologic characteristics? At present the answer is still more or less uncertain but rapidly growing knowledge regarding the influence of DNA upon RNA and protein synthesis supports earlier views (Beadle and Tatum 1941) that genes control the formation and the action of enzymes Since enzymes in turn control biosynthetic and other metabolic events (see p 40) control of enzymes can be regarded as tantamount to control over function and form As in higher organisms a given bacterial characteristic may be controlled by more than one gene (e g virulence and high resistance to certain antibiotics) or one gene may affect several traits (e g, see

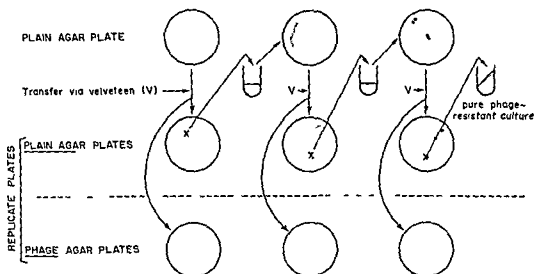


FIG 14 Diagram illustrating the Lederberg technic of indirect selection of mutants by replica plating The growth from a phage susceptible inoculum on plain agar is imprinted on velveteen which is then used for inoculating both plain and phage containing nutrient agar plates Following an appropriate period of incubation the area of growth on a plain plate corresponding to the site where a phage resistant colony developed on phage agar is picked Since this area contains not only one or a few phage resistant mutant cells but still is highly contaminated with phage susceptible cells an enrichment procedure is necessary in order that a pure culture of resistant cells may be obtained Therefore the isolate from the plain agar plate is transferred to plain nutrient broth incubated and an aliquot from this broth culture is used for inoculating plain agar The resulting growth now containing a larger concentration of phage resistant cells is then used for a repetition of the described replica platings This procedure is repeated several times and each time the location of the presumably phage resistant cell on plain agar is determined by inspection of the site of growth on replicate plates containing agar *plus* phage Following this process of *indirect selection it is possible* to obtain a pure culture consisting of stable phage resistant cells (Braun W Bacterial Genetics Philadelphia Saunders)

nal mutant cells or (3) the second generation offspring of 4 mutants etc One particular version of the fluctuation test can overcome such difficulties and permits the determination of mutation rates even under conditions where there is concomitant occurrence of new mutants and multiplication of mutants that arose in prior generations This modified fluctuation test derives its advantage from the fact that mutation rates can be determined on the basis of scoring whether or not a *single* mutational event has occurred in individual cultures of a large series of replicate cultures additional mutational events that might occur in any one culture are not taken into consideration in this particular procedure Table 2 shows a few examples of mutation rates that have been determined with the aid of the fluctuation test

So far the most direct demonstration of the undirected occurrence of mutational events has been contributed by the replica plating test of the Lederbergs This test is based on a technic of transferring bacteria from the surface colonies of one agar plate to a series of other plates with the aid of a velveteen stamp This technic permits members of one colony (i e members of one clone in the case of *E coli*) to be transferred to corresponding sites on plates with *entirely different media* for example media containing streptomycin phage or no selective supplements This in turn permits the indirect selection of pure resistant cultures from an initially predominantly sensitive population *without* placing the organisms in the course of selection into contact with the specific environment to which they prove to be resistant (see Fig 14) This procedure as stated represents the most conclusive method so far devised to demonstrate

was occurring during this period and involved only the descendants of a few tryptophan independent mutant cells Eventually, the resulting mutant clone or clones, attained sufficient size to contribute to readily apparent growth, measurable turbidimetrically The establishment of the vast majority of heritable changes occurring in bacterial population, including changes in resistance antigenic characteristics potential fermentative capacities, nutritional requirements etc , now have been demonstrated to involve similar replacement of the original population by the selective growth of mutant cells with increased fitness to the existing environmental conditions

Whereas these findings have revealed the constant occurrence of genetic changes in any bacterial population (actually, in a minute fraction of the population as far as the determinants of a given trait are concerned), they did not reveal whether or not such changes occurred independently of the existing environment However a number of experimental test systems designed to shed light on this important problem have indicated that mutational changes in bacteria are indeed undirected random events just as they are in higher forms

The first of these demonstrations was contributed by Luria and Delbruck (1943) with the aid of their so called fluctuation test This test is basically statistical in nature and in its simplest form measures the presence or the absence of mutant cells after a given period of incubation in a large series of replicate cultures, initiated with a sufficiently small inoculum, e g 100 cells per ml to assure the *initial* absence of mutants The test has been applied primarily, but not exclusively to measure changes involving the acquisition of resistance e g , to bacteriophage streptomycin, penicillin, etc In these cases the occurrence of mutants resistant to a particular agent was measured in a series of replicate cultures *free* of the agent the presence of the mutant cells was detected by subsequent transfer to an environment containing the particular inhibitory agent e g streptomycin The results have been in conformance with the interpretation that resistance arises and is transmitted to daughter cells, quite independently of the presence of the inhibitor Although subsequently more direct demonstrations of the environment independent origin of mutants became available the fluctuation test still is used to determine the rate at which mutants occur It is very important to recognize that this rate is not necessarily reflected by the frequency (number) of mutants in a population For example 16 streptomycin resistant mutant cells in a population could be indicative of either (1) 16 independent mutational events or (2) the first generation progeny of 8 origi

TABLE 2 EXAMPLES OF MUTATION RATES

CHANGE INVOLVED	SPECIES	MUTATION RATE* PER BACTERIUM PER GENERATION
R → S (colonial morphology)	*Salmonella aertrycke*	$5 \times 10^{-}$
Flagellar antigenic phase		
group → specific	*Salmonella typhimurium*	3×10^{4}
specific → group	*Salmonella typhimurium*	1×10^{5}
Radiation resistance	*E coli*	1×10^{5}
Threonine resistance	*Salmonella typhimurium*	4×10^{6}
Resistance to isoniazid	*M ranae*	3×10^{-6}
Penicillin resistance	*Staph aureus*	1×10
Resistance to phage T_1	*E coli*	3×10^{8}
Tryptophan independence	*Salmonella typhimurium*	5×10^{-8}
Histidine independence (h^- to h^+)	*E coli*	3×10^{-8}
Histidine dependence (h^+ to h^-)	*E coli*	1×10^{-6}
Streptomycin dependence	*E coli*	1×10^{10}
Streptomycin resistance (1 000 µg)	*E coli*	1×10^{10}
Streptomycin resistance (1 000 µg)	*H pertussis*	1×10^{10}

* For specific references see Braun 1953 p 63

Since with most antibiotics (with the notable exception of streptomycin and also of isoniazid) high resistance cannot develop in one step quick attainment of sufficiently high antibiotic concentrations can prevent the occurrence and establishment of resistant types

The *multigene* (or *polygenic*) control of high resistance to most antibacterial agents has been demonstrated not only on the basis of the mode of development of such resistance but also with the aid of recombination experiments (Cavalli and Maccacaro 1952) It will be recalled that in the process of recombination part of the genotype of one parent is exchanged for a corresponding part of the genotype of the other parent It has been shown that recombinants resulting from a conjugation between a highly resistant and a sensitive parent can show intermediate levels of resistance Also recombinants from two parents with intermediate resistance (controlled by unlike genes) can show very high resistance (e g $r_1r_2s_3s_4 \times s_1s_2r_3r_4 \rightarrow r_1r_2r_3r_4$ where r_1 etc denote genes controlling resistance and s_1 *etc sensitivity*)

In the case of a few antibacterial agents e g streptomycin and sulfadiazine mutational events leading from resistance to actual *dependence* upon the inhibitor have been observed One reason for such an odd phenomenon has been illustrated in the case of sulfadiazine dependence It was shown that the sulfa drug interferes with the utilization of the normal metabolite para aminobenzoic acid (PABA) In resistant mutants such interference can be reduced by an increased synthesis of PABA However certain mutational events can raise rates of PABA production to such an extent that the normal metabolite becomes toxic This toxicity in turn can be eliminated by permitting the antagonistic inhibitor sulfadiazine to exert its interference and to compensate in this manner for the toxic overproduction of the normal metabolite As a result these mutant cells become dependent upon sulfadiazine for growth

Relationships between altered bacterial resistance to phagocytosis and altered virulence have been studied to a considerable extent Such changes in resistance usually are associated with alterations in the characteristics of the bacterial cell surface Therefore they also tend to be associated with changes in antigenic properties since these too are dependent upon the nature of the cell surface (see below) Similarly the level of resistance of many bacterial pathogens to bactericidal factors present in blood tends to be correlated with virulence and antigenic properties However mutational changes in resistance to bactericidal blood factors may also occur independently of antigenic changes Recently an interesting *inverse* correlation between resistance to bactericidal serum factors and resistance to penicillin has been uncovered (Michael and Braun 1957) this means that genotypic and phenotypic changes affecting an increase in resistance to penicillin tend to be associated with corresponding decreases in bacterial resistance to inhibitory serum factors Since penicillin is known to interfere with the synthesis of certain components of bacterial cell walls the observed inverse correlation between penicillin and serum resistance may be ascribed to an alteration affecting the bacterial surface

Mutants with Altered Cellular Morphology Among the many known mutants with altered cellular morphology a particular group displaying a drastic alteration of cellular architecture has been recognized recently (Lederberg and St Clair 1957) It has been known for a long time that many bacteria can occur occasionally in a unique form in which the individual reproductive elements are round minute (often filterable) and frequently have been reported to originate from an initial giant structure (see p 37) The significance of these so-called L forms (*L* after the Lister Institute where they were first observed) remained obscure until Lederberg and others recently provided evidence indicating that they actually represent mutants deficient in their capacity to form cell walls This deficiency seems to be responsible for the unusual mode of reproduction by budding of these forms (Fig 8) their round shape and their long recognized high resistance to penicillin (which as mentioned above interferes with the synthesis of cell wall components) The L forms thus appear to be genetically stable counterparts of the cell wall-deficient viable structures (protoplasts) that can be achieved by exposing certain sensitive bacterial cells to penicillin plus 20 per cent

the spontaneous, undirected occurrence of the change (mutation) that endows the affected cell and its progeny with the new potential for resistance Most investigators today believe that this represents a typical model for the randomness (with respect to environmental influences) of all mutational events in bacteria

This randomness is not restricted to the occurrence of individual mutational events but also extends to the relationship among a series of mutational events that may affect an individual cell This means that the occurrence of one mutational event in a cell does not tend to produce a significant alteration of the probability for the occurrence of another mutational event (for an exception *cf* mutator gene p 64) This mutational independence can be illustrated by the fact that a cell likely to mutate to resistance to one antibiotic at the rate of 1×10^{-7} per cell per generation and to another antibiotic at the rate of 1×10^{-9}, is likely to undergo simultaneous mutations endowing it with resistance to both antibiotics at the rate of 1×10^{-16} In other words, the likelihood of simultaneous occurrence of two mutational events in *one* bacterium is the product of the probabilities for each of the mutational events Now an event as rare as 1×10^{-16} per cell per generation is most unlikely to occur in hosts or in the laboratory since under these conditions bacteria do not usually attain population sizes of this order This then is one of the principal reasons for the practice of employing simultaneous administration of two antibacterial agents in therapy Whenever mutations leading to resistance to each of these agents involve independent events 'combined therapy can dramatically lessen the likelihood of the in vivo occurrence of resistant strains In certain cases however combined therapy instead of having such desirable effects may produce antagonistic effects resulting from an interference by one antibacterial agent with the action of the other (Jawetz et al 1954)

Representative Mutant Types

In the following section we shall cite a few features of representative types of bacterial mutants with special reference to problems of relevance to infectious processes

Mutants Resistant to Inhibitory Agents * A wide variety of mutant types with increased resistance to ordinarily inhibitory environmental conditions have been isolated and studied Among them are mutants with significantly increased resistance to any one of the many antibiotics to dyes heavy metals bactericidal blood factors phagocytosis UV radiation, or to bacteriophages Altered resistance to bacteriophages is interesting in at least two aspects (1) mutation from sensitivity to resistance to *one* type of phage is not necessarily associated with resistance to other types of phage (2) the change from sensitivity to resistance can be an all or none' event which is consistent with the assumption that such a change reflects a single gene mutation Similar one step changes from sensitivity to total resistance are rare but include one other widely studied mutational event namely the one step change from streptomycin sensitivity to total streptomycin resistance In contrast high levels of resistance to most other antibiotics develop as the result of multistep changes This means that mutation of a single gene raises the resistance only to a limited extent (e g to 10 units of penicillin p r ml) another gene may mutate subsequently and the combined activity of the 2 genes can now increase resistance to a much higher level (e g to 100 units of penicillin) A third change would raise the resistance to still higher levels and in this manner optimum resistance will be the product of the influence of many genes each of which mutated at its own rate If as it has been found to be the rule each mutational step in this stepwise resistance increase is likely to occur with a frequency of as low as 1×10^{-9} the likelihood that two such steps may occur simultaneously raising the initial resistance of a bacterium to significantly high levels would be 1×10^{-18} nil for all practical purposes This fact underlies the recommendation that in antibiotic therapy efforts should be made to reach high in vivo levels of antibiotic concentration as rapidly as possible thus reducing the likelihood of maintaining suboptimal inhibitory concentrations that permit the propagation of first step and subsequently multistep resistant mutants

* For a detailed review see Bryson and Demerec 1955

colonial morphology virulence and antigenicity as well as immunogenicity If a mutation effects merely a decrease rather than a complete loss of the surface antigens so called intermediate (I) types result If in the case of gram negative bacteria a mutation causes loss of surface lipoproteins relatively avirulent nonimmunogenic rough (R) types result which give rise to rough appearing colonies (Fig 15) if a decrease in lipoproteins is accompanied by an increase in mucopolysaccharides so-called mucoid (M) types result which also are relatively avirulent and develop into sticky colonies of distinctive morphology In many gram positive bacteria the surface antigen of virulent types is predominantly polysaccharide in nature usually arranged in the form of a distinct capsule (see p 30) S → R mutation in these species is accompanied by a loss of the capsule loss of associated serologic specificity (type specificity) change in colonial form and loss of virulence It should be noted that some confusion in terminology exists due to the fact that in the case of gram positive bacteria some investigators refer to the virulent encapsulated types as the M type rather than the S type

Genetic changes affecting cell surface characteristics can cause associated changes in a number of other characteristics besides those so far discussed These additional characteristics include some that are also dependent upon surface structure Among the latter characteristics are resistance to bacteriophage or certain antibacterial agents cellular appearance and agglutinability by salt all of which have been found to be altered frequently in association with antigenic virulence and colonial changes However changes in virulence or colonial morphology may also occur by virtue of genotypic changes that do not affect the cell surface (for example virulence can be a function of reproductive rates in vivo or of the duration of the in vivo lag period see p 77) Therefore independent changes in these characteristics may also be expected to occur This is indeed the case and is illustrated by ample data on the occasional occurrence of *independent* changes in virulence and antigenicity in colony morphology and antigenicity or in colony morphology and virulence etc (cf Braun 1953)

Another type of independent antigenic variation is of considerable importance particularly for taxonomic purposes in the case of certain flagellated bacteria The presence of flagella enables motile forms to spread over the surface of solid agar media resulting in a film of growth that resembles the foggy condensation produced by breathing on a cool surface Therefore early German investigators labeled these flagellated types as H (from *Hauch*) forms In contrast nonflagellated nonspreading mutants were labeled O (*ohne Hauch*) Both flagellated and nonflagellated bacteria possess distinctive antigenic properties that can be detected by serologic procedures Thus flagellated cells possess both H (flagellar) and O (somatic) antigens Loss of the H antigen is associated with a loss of flagella and it is now recognized that the remaining O antigen corresponds to what is commonly labeled S antigen in other species a loss of O antigen therefore is comparable with an S → R change The flagellar H antigen itself can be subdivided into at least two serologically distinct antigens the so called group and specific antigens which have been studied with great intensity in *Salmonella* species Variation from group to specific and also from specific to group (so called phase variation) occurs at unusually high rates (e g $1 \times 10^{-}$) in *Salmonella* and recent studies have uncovered some of the complex genetic mechanisms responsible for these changes (Lederberg and Iino 1956)

Even prior to the development of the present concepts of the genetic basis of antigenic variation the serologic analysis of antigenic variants aided materially in the recognition of cellular components which were not detectable by cytologic procedures Immunochemical analysis of antigenic variants thus provided at least a partial cytochemical basis of the understanding of factors associated with pathogenesis and immunity (see Dubos 1945 for further details)

Nutritional Mutants Mutations resulting either in a loss or a gain of a nutritional requirement have been investigated widely by bacterial geneticists and biochemists Mutants that have lost the ability to synthesize an essential growth factor and therefore have become dependent upon the addition of this growth factor to the culture medium are

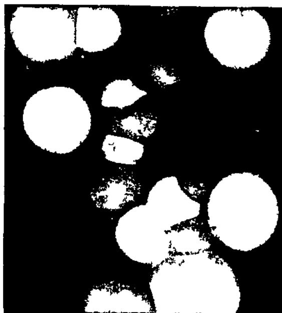


FIG 15 S and R type colonies of *Brucella abortus* on 2 per cent glycerol 1 per cent glucose nutrient agar (oblique lighting)

sucrose In the case of a few L type mutants it has been possible to detect the actual site of their deficiency by demonstrating that supplementation of the medium with diaminopimelic acid (DAP) an amino acid normally found in bacterial cell walls (p 33) will temporarily convert L type cells into normal walled bacteria As soon as DAP is removed from the medium these bacteria will revert to the L type

The possible significance of such mutant types in infectious processes has not yet been elucidated However they might prove to be important because local hypertonicity in tissues may keep these wall deficient forms alive and reproductive in vivo whereas the absence of similar hypertonicity in the usual artificial growth media may interfere with the recovery of such forms from infected hosts

Mutants With Altered Colonial Antigenic and Virulence Characteristics Strictly speaking many of the mutants falling into this category also represent mutants with altered cellular morphology As already mentioned under the discussion on resistant mutants, alterations in cell surface characteristics including actual deficiencies in wall materials tend to alter antigenic and (in the case of pathogenic species) virulence* properties of the affected bacteria In addition these surface characteristics usually also affect the manner in which cells arrange themselves spatially in relation to each other during colony formation on solid media Thus colonial morphology antigenicity and virulence tend to change simultaneously, a fact which has long been recognized and, at one time gave rise to the belief that these associated changes represent part of a definite, regularly recurring life cycle Early investigators even invented a special name for this phenomenon they referred to it as dissociation and to the various antigenic and colonial types as phases With the advance in knowledge of bacterial genetics came the now generally accepted recognition that the entire phenomenon of changes in antigenic colonial and virulence properties is merely another instance of spontaneous mutation involving cell surface properties and the subsequent selection of such mutants under appropriate environmental conditions (Braun 1947) However much of the terminology of the past has been carried over into the present

Thus it has long been recognized that a smooth (S) colonial type tends to be associated with cells that possess either a lipoprotein surface (in the case of gram negative bacteria) or a surface rich in polysaccharide and proteins (in the case of gram positive bacteria) These surface characteristics in turn endow such cells with certain properties (including increased resistance to phagocytosis) that are characteristic of bacteria of high virulence† The actual chemical composition of these surface components can differ within species and among species these differences can be detected by serologic methods and thus permit subgroupings into serotypes (type specificity) Moreover cells with these surface components tend to stimulate the production of specific protective antibodies in their hosts A change in these cellular surface properties can lead to simultaneous changes in

* Actually it is not entirely proper though customary to refer to *the virulence of a bacterium* since virulence is a function of interactions between host and parasite and thus is governed by properties of the bacterium *and* the host

† Cf Dubos 1954

descendants the result is a gradual population change involving the establishment of the mutant type Naturally when the selective value is in favor of the parent type cells population changes will fail to occur Occasionally as in the case of antibiotic resistance environmental conditions may endow the mutant cell type with an absolute selective advantage over the parent cells in these cases a complete and sudden shift in the composition of the population can occur As a rule however population changes particularly in regard to traits affecting virulence are of a more gradual nature In rare instances both mutant type cells and parent type cells may have equal selective values i e one will grow as fast as the other in the mixed population In such cases the eventual composition of a population will be determined solely by the rate of mutation to the new genotype balanced by the rate of mutations in the opposite direction re establishing the original phenotype Such interactions due to mutation pressure resulting in the continued coexistence of two distinct types within a population have been observed in the case of phase variation (p 75) where the selective values of different antigenic types can be identical under constant environmental conditions (Stocker 1949)

Truly constant environmental conditions are rarely encountered in nature or during laboratory cultivation of bacteria Usually the metabolic activities of the bacteria or the response of a host to their presence will result in a gradual alteration of the bacterial environment In vitro such alterations can be minimized with the aid of special laboratory devices that have been developed to maintain continuous growth of bacteria under conditions where the environment (liquid nutrient medium) is constantly renewed (see review by Novick 1955) In such continuous culture devices (e g turbidostat chemostat) the number of bacteria per milliliter of medium can be held constant and the excess bacteria (and spent medium) are removed automatically One important finding from studies with such *open systems* is that continuous growth (in association with selection pressure that is different from that commonly encountered in closed systems) can lead to the periodic establishment of mutants with greater and greater selective advantage (periodic selection Atwood et al 1951) For example investigations with continuous growing *Salmonella typhimurium* populations revealed the establishment of antigenically identical mutants with significantly shorter generation time about every 200 generations Since such faster growth rates should they occur in vivo would provide pathogenic strains with increased advantages over host defenses it is quite possible that periodic selection may contribute to gradual increases in virulence known to occur in many epidemics In complete contrast with such open systems are the usual methods of laboratory cultivation of bacteria in test tubes or flasks Such cultivation involves *closed systems* in which exhaustion of nutrients accumulation of metabolites gradual alterations in pH and oxygen tension contribute to constantly changing environmental conditions and corresponding alterations of selection pressure Therefore in such closed systems the fate of mutants not only can differ strikingly from their fate under more natural (and more open) conditions in vivo but also may differ depending on whether a mutant arises early or late in the course of the culture s growth

These differences have been illustrated amply by observations on dissimilarities between in vitro and in vivo population changes of pathogenic bacteria Whereas in susceptible hosts bacterial population changes are such that the selective establishment of more virulent immunogenic mutant types is favored (e g non S → S) in vitro population changes tend to be in the opposite direction (S → non S) An analysis of factors contributing to the S → non S population changes occurring in vitro has revealed that the selective advantage of the antigenically different less virulent non S mutants can be referred to their greater resistance to inhibitory metabolic products accumulating in the culture medium (Braun 1956) Among such metabolites are certain D-amino acids which can be produced by the parental S cells and inhibit their continued reproduction whereas spontaneously arising non S mutants possess a higher level of D-amino acid resistance and thus can establish themselves selectively Interference with the production or the accumulation of such differentially inhibitory metabolites has been

called *auxotrophs* their parental cells which do not need this growth factor are referred to as *prototrophs* Mutations involving changes from auxotrophy to prototrophy, as in the case of tryptophan requirement (discussed on p 69) also occur All sorts of auxotrophic mutations have been detected including mutations establishing needs for specific amino acids, vitamins, purines or pyrimidines (cf Wagner and Mitchell 1955) Special procedures for the isolation of such mutants have been developed and the specific mutants thus isolated, in turn, have been extremely useful in the biochemical analysis of the actual steps involved in the biosynthesis of numerous compounds (cf Vogel and Bonner, 1958)

This usefulness of auxotrophic mutants is connected with the fact that they exhibit characteristic interruptions (or blocks) in particular pathways and these blocks may have analyzable consequences A particularly helpful situation arises when mutants can be isolated that are (individually) blocked at different steps within a given pathway With the aid of such mutants it is often possible to establish the sequence of intermediates within a pathway

Certain types of auxotrophy have been shown to exert a significant effect on host parasite relationships Thus mutation to purine requirement has been found to reduce the virulence of *Klebsiella pneumoniae Salmonella typhimurium* and *Pasteurella pestis* for mice Apparently the purine concentration in such hosts is insufficient to support the multiplication of these otherwise virulent organisms injecting or feeding infected mice with purine re establishes the virulence of these auxotrophs It also has been noted that purine auxotrophs produced different virulence effects, depending upon the time of year when they were tested in mice These apparently seasonal differences in virulence eventually were found to be attributable to seasonal differences in the purine content of the diet of the mice used in these studies The relationship of such observations to certain seasonal alterations in the severity of some infectious processes remains to be established *

Pathogenic bacteria often possesses fairly complex nutritional requirements This may be attributed partly to the abundant supply of nutritional factors in their host environment which can easily support the growth of exacting strains It has been suggested (cf Lwoff 1944) that such fastidiousness of pathogens may have arisen as a result of mutational ' losses of function ' of these organisms during their evolution

Fermentation and Pigmentation Mutants Due to the relative ease of their identification fermentative characteristics and pigment formation have been used widely for taxonomic purposes Both types of characteristics have long been known to be subject to considerable variation which now is recognized to include both phenotypic (temporary modification) and genotypic (mutational) changes The rates of mutation for these particular traits tend to be fairly high, usually in the neighborhood of 1×10^{5} to 1×10^{-7} per bacterium per generation As previously mentioned in the section on phenotypic alterations when a strain that has lost the capacity to ferment a specific carbohydrate substrate regains this capacity by mutation the latter genotypic change may merely set up the *potential* for such cells and their descendants to carry out such fermentation The realization of this potential may depend on enzyme induction (see p 62) In other words the mutational event itself does not lead to an altered phenotype (formation of the specific enzyme and subsequent utilization of the carbon source) until the specific environment (carbohydrate) is present Such a distinct separation between the basic genetic change and its subsequ nt phenotypic manifestation is not necessarily confined to fermentation mutants but is not always readily demonstrable

Population Changes

Mutations occur in individual cells in a population of bacteria the subsequent fate of a genetically altered cell depends upon its fitness in relation to that of the parental cells to survive and to propagate under the existing environmental conditions In the resulting competition between mutant cells and parent cells the differences in their respective selective values can be slight If the difference is in favor of the mutant cell its progeny will gradually outgrow the parental cells and their

* Detailed discussions on the influence of nutritional requirements of bacteria upon host parasite relationships have been contributed by Garber 1956

conditions unfavorable for growth (e g 5 C) and infrequent transfers of cultures are important for the maintenance of relatively stable populations Much has been learned in recent years about the artificial alteration of selective forces in vitro which permits a certain amount of control over undesirable population changes (Braun, 1953) Such control is frequently of considerable importance to problems associated with medical bacteriology the retention of a desired immunogenic type during vaccine production is an outstanding example

CLASSIFICATION OF BACTERIA

The type of variability that has been discussed for individuals and cultures obviously also must have played a role and presumably still plays a role in the evolutionary history of bacteria As has been indicated in the discussion of morphologic and physiologic characteristics the micro-organisms grouped under the label of bacteria represent an exceedingly diverse array of form which may to some extent be indicative of their diverse evolutionary origin It is the task of taxonomy i e the science of classification to arrange such diversity into distinct groupings This may be done with the aid of an appropriate scheme or determinative key based on easily identifiable characteristics which can help to assemble and to subdivide large groups of organisms into easily identifiable subgroup However such artificial groups do not necessarily reflect natural or evolutionary relationships among their members therefore taxonomists dealing with higher organisms have arrived at so-called natural schemes of classification reflecting such relationships Plant and animal taxonomists were aided by the fact that in general sufficiently closely related higher organisms are actually or potentially able to *interbreed* Thus they were able to define natural delineations among species by determining the existence of fertility barriers In the absence of sufficient information on the frequency of occurrence of sexlike mechanisms among bacteria it has been impossible to establish a comparable system of classification for these micro-organisms Artificial schemes of bacterial classification which are indispensable for diagnostic purposes initially were based upon differences in morphologic characteristics (shape of the cells presence of spores or flagella) Later systems added physiologic and biochemical traits as important differentiating criteria All these systems employed the same labels for their groupings as those used for higher organisms (strains species 'genera tribes families, etc) but it should be clear that the species concept in bacteriology does not correspond too closely to the one applied to higher organisms Among the latter species were largely established on the basis of criteria reflecting natural relationships whereas the bacterial species represent a more arbitrary assemblage of similar forms Since the criteria for recognizing bacterial species are thus more diffuse, the delineation of bacterial species sometimes seems to be too broad and sometimes too narrow For example many so-called species of *Salmonella* can be regarded as mutant types (serotypes) which can be shifted from one species to another by either mutation or transduction In many cases organisms now recognized as belonging to a single species have been described under a number of different names because they were observed in several variant forms Such confusion is quite understandable when it is realized that the characteristics commonly employed for taxonomic criteria of bacteria include many that are known to mutate at particularly high frequencies (fermentative capacities pigmentation antigenic characteristics) In view of such variability it may appear surprising that useful taxonomic schemes can be devised at all and that usually (but not invariably) natural isolates conform to textbook descriptions One reason that this is possible and that members of a taxonomic group tend to display a rather constant combination of definable characteristics lies in the high survival value of specific genotypes under more or less specific environmental conditions This means that organisms possessing a certain combination of traits will be favored in their natural environment over an occasional mutant type displaying alterations of these traits To illustrate a combination of certain characteristics including sensitivity to thionin dyes and requirement for CO_2 have been used to define the causative bacterial agent of abortion in

shown to prevent population changes In a number of aerobic pathogenic species an increase in available oxygen also has been found to prevent S → non S population changes in vitro In general population changes in vitro are less likely to occur in solid media compared with liquid media part of the underlying cause for such population stabilization resides in the spatial restrictions upon mutant clones on solid media and in an interference by such media with the selective effects of many metabolites Until quite recently, it has been extremely difficult to duplicate in vitro the usual direction (non S → S) of population changes occurring in vivo However studies that so far have been limited to trials with members of the genera *Brucella Diplococcus* and *Salmonella* (see Braun 1956) have shown that non S → S population changes can be produced in liquid laboratory media provided that small amounts of DNA (from various bacterial or animal sources) and the depolymerizing enzyme DNase are added To what extent naturally occurring DNA breakdown products may perform similar selective functions in favor of virulent types in vivo (in addition to the selective effects produced by phagocytes bactericidal blood factors etc) remains to be established DNA breakdown products undoubtedly are present wherever cellular or bacterial destruction takes place in vivo and the administration of such material to mice infected with pneumococci has been shown to enhance the rate and the incidence of host mortality (Braun et al 1957)

Studies of both experimental and natural infections have shown that susceptible hosts tend to produce strong selective effects in favor of virulent types However relatively avirulent mutants have been isolated quite frequently from chronic stages of bacterial infections and bacterial population changes involving the selective establishment of less virulent mutants have been noted when mixtures of S and non S types were injected into resistant species or resistant stages of a given species (e g chick embryos vs hatched chicks) In addition selection in opposite directions may occur in different locations of individual hosts For example in experimental *Brucella* infections of guinea pigs it has been observed that relatively avirulent non S mutants occasionally can be isolated from enclosed abscesses of animals infected several weeks earlier with virulent S bacteria at the time of isolation of such avirulent mutants the same animals yield virulent S type cells from other tissues such as spleen, liver and lymph nodes The presence of non S types in the closed abscesses of these animals may be attributed partly to the high local metabolite concentration known to favor these mutants and partly to the absence of strong S favoring selective influences such as those produced by bactericidal blood factors This and many other findings suggest that, in general host susceptibility is closely associated with the tendency for in vivo selection of highly virulent bacterial types whereas host resistance including chronic stages of some infectious diseases tends to be associated with selective in vivo conditions promoting the establishment of less virulent mutants

The fate of bacterial mutants and species in vivo is not only dependent upon the influences exerted by host components but also upon effects produced by the presence of other bacterial species Such ecologic effects for example are quite pronounced in the case of the intestinal bacterial flora and can be illustrated by the finding that usually inconsequential exposures of mice to *Vibrio cholerae* can be converted into exposures with lethal effects following the reduction of the intestinal flora by suitable antibiotic treatment (Freter 1955)

Primary Isolations and Maintenance of Cultures

The existence of specific selective forces in vivo and the usual absence of similar forces under in vitro conditions demand special caution in the isolation of pathogens from infected hosts If it is important to preserve under in vitro conditions the genotype that existed in vivo care must be taken to prevent population changes This means that wherever possible prolonged cultivation in liquid media particularly in the presence of immune sera which are known to enhance population changes should be avoided Growth on solid media is preferable since it is less likely to support rapid population changes Also since each growth period provides additional opportunities for the occurrence of mutants and their selection preservation of cultures under

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cattle, *Brucella abortus*, a related form usually isolated from swine, *Brucella suis* is thionin resistant and does not require CO for growth However, this does not mean that intermediate forms, displaying thionin resistance and CO requirement cannot occur they do occur under laboratory conditions and, with extreme rarity in nature Also a thionin resistant CO independent type can be isolated occasionally from cattle However, as a rule it is the genotype producing thionin sensitivity and CO requirement that attains greatest survival value in cattle and, therefore is recognized most frequently in primary isolates from these animals Thus in many instances what is described as a distinct species (e g *B abortus* or *B suis*) may profitably be regarded as a *biotype*

Despite their seemingly arbitrary origin schemes of bacterial classification based on morphologic and physiologic characteristics appear to reflect to some extent natural (evolutionary) relationships For example recent studies on transformation among members of the genus *Hemophilus* have revealed that the efficiency of genetic transfers among member species is correlated with their arrangement in current taxonomic schemes (note however that here is another case of interbreeding among so-called species) On the other hand recent observations such as those revealing the occurrence of recombinations and transductions involving *Escherichia* and *Shigella* certainly indicate that the present generic differentiation between these organisms represents an unnatural classification

More natural classifications for bacteria might be obtained by taking into consideration compatibility for genetic transfers and the genetic plasticity existing within bacterial groups For an adequate representation of this plasticity a description of all types occurring within the potential mutational range of the species to be defined would be required This however would meet with grave practical difficulties therefore we have to restrict ourselves, at least for the time being to certain useful classifications which are based on the cataloguing of certain morphologic and physiologic traits but nevertheless exhibit some aspects of a natural system The most widely used of these classifications is set forth in Bergey s *Manual of Determinative Bacteriology* (Bergey 1957) Among the characteristics used in this classification are cellular and colonial morphology including the presence and the location of spores and flagella the method of cell division staining characteristics metabolic characteristics, particularly nutritional competence performance of easily detectable enzymatic activities immunologic specificity and pathogenicity e pecially in regard to host range As stressed repeatedly during the preceding discussion, all of these traits are subject to both genetic and environmental modifications Therefore, th intelligent utilization of taxonomic schemes requires the recognition that well defined environmental conditions are needed for comparative descriptive studies Furthermore the lack of biologic definitions imposes limitations regarding the exact taxonomic lines at which a distinction among mutant forms as progenitors of strains ends and a separation into species begins

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4

Pathogenic Properties of Bacteria and Defense Mechanisms of the Host

The purpose of the author in this chapter is to discuss examples of factors possessed by bacteria that enable them to cause disease and to describe protective mechanisms of the host Much has been written in philosophic vein about commensalism and parasitism between micro organism and animal or plant host It has been stressed that disease is a rare event in the normal para itic relationship and there fore is a minor aspect of parasitism in which peaceful coexistence is averred to be the rule On the other hand depression of the host s resistance by ionizing radiations for example shows clearly that bacteria which we are wont to consider as commensals the normal flora of the gut under usual circumstances are pre vented from invading the tissues to cause dis ease and death only because the protective mechanisms are intact Furthermore many of the species of micro organisms that cause disease are not found in a benign parasitic relationship as for instance the viruses of influenza measles and smallpox in man or rinderpest in cattle When these agents have been introduced into a population overt dis ease is the rule unless specific prophylaxis by immunization has been practiced or the popu lation has become immune because of previous epidemic infection Between the two extremes of parasites which usually are commensal and those that usually cause widespread disease in the unimmunized is a spectrum of relation ships in which the frequency of disease de pends on an unsteady balance between the state of the host and the pathogenicity of the numberless microbial species or strains that are encountered

From the standpoint of the host the aim is to preserve homeostasis and a variety of pro tective mechanisms are available to achieve this end Homeostasis can be maintained de spite the presence of large and varied micro bial populations provided they are restricted to certain regions of the body where they can be tolerated However if for one reason or another there is a defect in the homeostatic barriers and the micro organisms gain access to other tissues disease results The relation between microbe and host is dynamic whether the species of micro organism is classed as pathogen or commensal The host appears to do everything in its power to rid itself of the parasite and if this is not possible at least to restrict it to a region where it can do the smallest amount of harm or occasionally be of benefit because the parasite is uniquely able to synthesize nutritional factors neces sary for the host or possesses enzymes ca pable of cleaving ingested materials to a state in which the host can utilize them

In summary it can be said of the relations between host and micro-organism that in some instances there is a state of relatively peaceful coexistence or commensalism in others there is an uneasy truce that is commonly broken and in the case of highly pathogenic microbial

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In summary it can be said of the relations between host and micro-organi m that in some instances there is a state of relatively peaceful coexistence or commensalism in others there is an uneasy truce that is commonly broken and in the case of highly pathogenic microbial

species the usual state is open warfare What ever military figure of speech is used to de scribe the complex relationships it is clear that no single generalization can apply to all, but that each case needs to be analyzed separately as done in subsequent chapters

Part 1

PATHOGENIC PROPERTIES OF BACTERIA

The term *pathogenicity* refers to the capac ity of micro organisms to cause disease, either natural or experimental, in a given host spe cies Pneumococcus is pathogenic for the mouse although spontaneous pneumococcal disease never has been observed in this spe cies Nonetheless the mouse is extremely sus ceptible to experimental infection and rapidly succumbs following injection of only a few bacteria In order for disease to occur natu rally in a population pathogenic bacteria must be endowed also with certain attributes which will be discussed under the collective term *communicability* although it should be borne in mind that factors pertaining to the host or to intermediate vectors in addition to intrinsic properties of the microbes play a large part in transmission of infection from one indi vidual to another

Pathogenicity and virulence have commonly been used as synonyms but as Miles (1955) nas pointed out a useful distinction can be made between the terms Pathogenicity may be regarded as a general attribute of a species or genus or some other grouping of micro organisms the term *virulence* being used to indicate the degree of pathogenicity of a given type strain or clone as observed in a particular host species under defined conditions *Bacillus anthracis* is pathogenic for many genera of animals Mutant strains or variants of anthrax bacillus show great differences in virulence or relative pathogenicity when tested in guinea pigs for example and the differences in viru lence are stable characteristics of the variants These then are virulent or avirulent strains of the pathogenic species, *B anthracis* Patho genicity of pneumococcus as a species is at tributable to the presence of antiphagocytic polysaccharide capsules which surround the cells In the same animal species mouse enor mous differences in virulence occur as charac teristics of different caspular types Moreover within a single type stable mutants exhibit high or low virulence correlated with synthesis of large or small amounts respectively of cap sular polysaccharide

Although the virulence of a specified stable strain of a microbial species can be measured with relative reproducibility in a given inbred strain of laboratory animal it is commonplace to find quite different results when other strains of the same animal species are simi larly tested It follows that the results found in one animal species should not be extrapo lated to another

Virulence can be expressed only in com parative terms because of variations in both host and parasite To arrive at even a rough numerical value for the virulence of a newly isolated micro organism be it bacterial, viral or fungal requires the use of large numbers of animals infected with increments of dosage and whenever possible by comparison with the results found with a calibrated strain of the same species tested at the same time Then a numerical comparison of virulence for the ani mal species under test can be made based either on the number or the dose of infectious agents required to produce a pathologic effect or else by differences in the effect caused by similar doses of each microbe

The nature of the effect chosen as the indi cator of virulence may vary from the number of micro organisms or colony producing units found per unit weight of a selected infected tissue to an estimate of the number and the extent of the lesions produced Death of the animal usually provides the most secure end point

The concept of a minimum lethal dose (M L D) of micro organisms as a measure of virulence may be grossly misleading because it does not take account of variations in resist ance of different members of the host popula tion of variation in virulence among the micro organisms inoculated nor of differences in response to increments of dose Death rates resulting from constant increments of dose in similar groups of a population of animals are found usually to increase slowly in the range of 1 to 10 per cent and 90 to 99 per cent mortality and most rapidly in the region of 50 per cent mortality For this reason the dose that kills 50 per cent of the animals can be

estimated more precisely than at either extreme of the mortality curve. As a result the median or 50 per cent lethal dose (LD_{50}) is used most commonly in the description of virulence. Calculation of the LD_{50} can be made by employing a formula such as that devised by Reed and Muench (1938).

In infectious processes where death cannot be used as the endpoint, other pathologic effects can be related to the dosage of microbes required to produce them in 50 per cent of the test animals in a given time. This dosage is often referred to as the median or 50 per cent infectious dose (ID_{50}).

Although estimation of the median lethal dose of certain well studied strains of microbes can be made in the laboratory with fair reproducibility, it should be emphasized that this measurement may bear little relation to virulence of a species of bacterium in the natural state.

MICROBIAL FACTORS THAT DETERMINE PATHOGENICITY

The production of disease involves interaction between microbe and host, and for this reason discussion of the pathogenic properties of the infective agent requires also a consideration of factors of host resistance. Recognition that bacterial products are concerned in virulence depends upon the specific pathologic effects they produce as well as on the immunologic response of the host to the infective agent or fractions of it.

Bail and Weil (1911) introduced the word "aggressins" to describe nontoxic factors produced by *B. anthracis* and other bacteria that interfere with host resistance and cause the production of specific protective antibodies. The usefulness of the term is debatable. The same may be said for the expression "aggressive factors," which have been defined as substances produced by micro-organisms that assist them to establish or extend the infection by inhibiting phagocytosis, by breaking down mechanical barriers to spread or by killing the host's tissues. The expression "aggressive factors" is so comprehensive that it could almost be used as a synonym for "pathogenic factors." However, because of the confusion that might result, the terms "pathogenic factors" or "virulence factors" will be used in the present discussion.

It should be understood clearly that the factors which govern the pathogenicity of one bacterial species may be entirely different from those of another species. This may be illustrated by contrasting the factors concerned in the pathogenicity of *Clostridium tetani* and pneumococcus.

If the spores of *Cl. tetani* are introduced into a wound together with some material capable of causing local tissue damage, germination takes place and growth of the vegetative form of the bacillus occurs locally. During growth a highly poisonous and freely diffusible protein, tetanus toxin, is elaborated by the bacteria. The toxin travels centripetally along peripheral nerves, probably in the interneuronal tissue spaces (see Wright 1955), reaches the central nervous system, where it affects the motor neurons and produces the frequently fatal disease, tetanus. The bacteria themselves are present only at the original site of introduction. All the characteristics of the natural disease can be reproduced in animals by injecting minute doses of highly purified tetanus toxin. Immunization by means of toxoid prepared from the toxin prevents the disease. *Cl. tetani* possesses a single factor, tetanus toxin, which appears to account for its pathogenic qualities.

The course of events differs greatly when a few virulent pneumococci are introduced into the tissues of a susceptible animal. The microorganisms multiply rapidly, invade the blood stream and are carried throughout the body. At death they are found in large numbers in all organs in striking contrast with the animal infected with tetanus bacilli. The capacity to invade and multiply in the tissues is determined by the polysaccharide capsule which surrounds virulent pneumococci. This viscous substance inhibits ingestion of the microbes by phagocytes, apparently in a mechanical way, and is not toxic for the isolated phagocytes or for the intact animal even though injected in very large amounts. No toxin in any way analogous to tetanus toxin is known to be produced by pneumococcus. Furthermore, antibodies to the purified polysaccharides confer complete protection against infection.

Therefore, the single demonstrable pathogenic factor in pneumococcus, the capsular polysaccharide, differs entirely from tetanus toxin both in its nature and mode of action and the mechanisms by which these two bac

Table 3 Factors That Influence Pathogenicity of Some Bacterial Species

Bacterial Species	Nature	Site of Action	Mode of Action	Specific Immunity to Infection	Comment
Pneumococcus	Capsular polysaccharide	Phagocytes	Inhibits phagocytosis	Antipolysaccharide antibodies	Antibodies enhance phagocytosis No significant toxin identified
Hemophilus influenzae	Capsular polysaccharide	Phagocytes	Inhibits phagocytosis	Antipolysaccharide antibodies	Role of endotoxic factors unknown
Streptococcus pyogenes	M proteins at cell surface	Phagocytes	Inhibits phagocytosis	Anti M protein antibodies	Hyaluronic acid is not antigenic Antibodies to various toxins do not prevent infection but may mitigate it eg antierythrogenic toxin prevents rash of scarlet fever antistreptokinase inhibits digestion of fibrin by streptokinase plasminogen plasmin system
	Hyaluronic acid capsule	Phagocytes	Inhibits phagocytosis		
	Numerous exotoxins and extracellular enzymes	Multiple blood vessel red blood cells leukocytes myocardium etc	Cytotoxic hemolytic various hydrolytic enzyme reactions		
Bacillus anthracis	D polyglutamic acid capsule	Phagocytes	Inhibits phagocytosis		Antibodies to capsular material not protective The precise relationships of the exotoxin to the immunizing antigen have not been clarified
	Extracellular toxin lipoprotein (?)	Generalized	Damages phagocytes produces edema and necrosis shock oligemia and death	Antibodies to toxin or possibly to spontaneously toxoided derivatives	
	Intracellular toxin lipoprotein (?)		Anticomplementary		
Pasteurella pestis	Envelope or capsular antigen (Fraction 1) carbohydrate protein complex	Phagocytes	Inhibits phagocytosis	Antibodies to capsular antigen *and* VW antigen complex	Antibodies to Fraction 1 protective for rats mice and monkeys but not for guinea pigs antibodies to VW protect mice against infection by virulent strains that lack capsular antigen
	VW antigen complex				
	Extracellular toxin protein (?)	Phagocytes	Inhibits phagocytosis and damages phagocytes		
Corynebacterium diphtheriae	Exotoxic protein	Cells of most tissues	Necrotizing inhibits oxidative processes possibly through interference with cytochrome b	Antitoxin	Toxin produced following infection with appropriate lysogenic diphtherial bacteriophages
Clostridium tetani	Exotoxic protein	Motor neuron in cerebrospinal axis	Anticholinergic(?)	Antitoxin	

Table 3 Factors That Influence Pathogenicity of Some Bacterial Species (*Continued*)

Bacterial Species	Nature	Site of Action	Mode of Action	Specific Immunity to Infection	Comment
Clostridium botulinum	Exotoxic protein	Cholinergic nerve ending in peripheral autonomic and somatic fibers	Anticholinergic inhibits release of acetylcholine	Antitoxin	4 major types of *Cl botulinum* A B C D produce immunologically distinct toxins
Clostridium welchii	Lecithinase exotoxin	Lecithin of cell membranes and mitochondria	Cytolytic interferes with succinoxidase activity of mitochondria	Antilecithinase	
	Collagenase	Collagen framework of intact tissues	Proteolytic		Destroys collagen framework in intact muscle Antibodies to collagenase and hyaluronidase not protective
	Hyaluronidase	Intercellular ground substance	Depolymerizes hyaluronic acid		
Salmonella typhosa	O antigens polysaccharide protein phospholipid complex	Circulating phagocytes and RES cells serum factor small blood vessel	Inhibits phagocytosis reduces properdin level inhibits complement modifies fibrinogen potentiate effects of epinephrine	Antibodies to O antigen and Vi antigen (?)	Nature of specific immunity to natural disease not defined
	Vi antigen (polysaccharide)	Phagocyte	Antiphagocytic		

terial species cause disease are quite different Tetanus is a highly fatal disease caused by a toxic metabolic product of a micro organism incapable of multiplying in healthy tissue whereas in pneumococcal infections the invasive bacteria cause death only after extensive multiplication in tissues of the host

Many diseases caused by bacteria fall into either one of the two categories illustrated by the foregoing examples However a larger number belong to a third category in which the micro organisms exhibit multiple pathogenic qualities as shown in Table 3

Possession of an antiphagocytic component such as a capsule does not mean that this material is the most significant factor in virulence This is well illustrated by the anthrax bacillus in which all naturally occurring virulent strains are found to be encapsulated on the other hand encapsulated strains may be avirulent It is apparent that other factors have overriding importance as compared with the capsule

To determine precisely the part that a particular cell component plays in the pathogenicity of a bacterial species may be very difficult In pneumococcus the situation is deceptively simple since invasiveness can be shown by a variety of methods to depend upon a single morphologic structure the polysaccharide capsule which is antigenic Moreover complete protection against infection is provided by antipolysaccharide antibodies In general demonstration that antibodies to a cell fraction are protective has afforded the most secure basis for inferring that it determines or influences pathogenicity In cases such as the anthrax bacillus in which antibodies to the polyglutamic acid capsule do not prevent infection more indirect methods must be used such as the demonstration in vitro that the capsule inhibits phagocytosis In Group A and Group C streptococci the hyaluronic acid capsules are not antigenic but appear to influence pathogenicity because it has been shown that infected animals may be afforded a degree of protection if they are injected with the enzyme hyaluronidase which digests away the hyaluronic acid capsule in vivo

If antibodies to a cell product prevent infection this is very strong evidence that it is a virulence factor For example antibodies to the M proteins on the surface of Group A streptococci are protective However, it is much more difficult to specify the role of other cell products such as toxins or extracellular enzymes of streptococci in the infectious process because antibodies to them do not prevent disease although they may modify its course Demonstration that an isolated product is toxic such as the O hemolysin of several bacterial species, cannot be taken to mean that it has significance as a determinant of pathogenicity At the same time one should not infer that such products have no influence on the course of disease on the grounds that antibodies to them do not prevent *initiation* of infection It would seem to be more reasonable to consider the M proteins as the primary determinants of virulence and the other cell products as secondary candidates that may come into operation after infection has been established

The species listed in Table 3 have been chosen to exemplify the multiplicity of factors that are involved in the pathogenicity of bacteria and to indicate something of their nature and mode of action Complete information is not available for any species Even in the case of pneumococcus which has been studied more extensively than any of the other species shown in Table 3 we are entirely ignorant of the factor or factors produced by it which cause death of the infected host It is abundantly clear that the nontoxic polysaccharide capsules are of primary importance in enabling pneumococcus to be invasive and equally plain that death of the host cannot be ascribed to a direct toxic action of the polysaccharides

1 *Nontoxic antigenic surface components which act by inhibiting phagocytosis and antibodies to which are protective* The polysaccharide capsules of pneumococci already referred to are examples of this category Through inhibition of phagocytosis they permit multiplication of bacteria in the tissues Antibodies to them promote phagocytosis and protect against infection Many other important pathogens such as *Hemophilus influenzae* and *Klebsiella pneumoniae* likewise owe their chief pathogenic activity to the protection against phagocytosis afforded by antigenic polysaccharide capsules

Proteins on the bacterial surface may act

in a similar way Group A streptococci (*Streptococcus pyogenes*) can be differentiated into many types based on immunologically distinct acid soluble M proteins which are present at the cell surface Although Group A streptococci produce many other components toxins and enzymes which may play a path ogenic role once infection has been established the ability to initiate the disease process depends in the main on the antiphago cytic activity of the M proteins Moreover anti M antibodies are protective whereas antibodies to other cell products although they may modify the disease do not prevent infection

Naturally occurring fully virulent *Pasteurella pestis* produces a surface material often referred to as the envelope antigen capsular antigen or Fraction 1 which inhibits phagocytosis It has been isolated as a carbohydrate protein complex and antibodies to it afford protection against infection in mouse rat monkey and presumably in man but not in the guinea pig (for review see Burrows 1955) Burrows (1957) has described a second antiphagocytic material termed the VW antigen complex which also enables the microorganisms to resist phagocytosis and is toxic for leukocytes Mutants of the plague bacillus occur which are virulent for mice but lack the capsular antigen These mutants owe their virulence to the antiphagocytic toxic VW complex which presumably is disposed at the cell surface Serum containing antibodies to Fraction 1 and to VW protect mice against infection by noncapsulated VW containing strains but anticapsular antibodies alone do not However virulent strains as normally encountered possess both the capsular and the VW antigens and at least in experimental infections of mouse rat and monkey antibodies to both antiphagocytic materials do not appear to be necessary for protection but only the anticapsular antibodies However a maximum degree of protection may require both antibodies Discovery of the VW antigens provides an explanation for the older observations that although fully virulent wild type *P pestis* are encapsulated not all encapsulated strains are virulent It may be that the latter lack the VW antigens

2 *Nontoxic antigenic surface components which inhibit phagocytosis but antibodies to which are not protective* All pathogenic strains of *B anthracis* form capsules composed chiefly of D polyglutamic acid which has antiphagocytic properties However capsulated but nonvirulent strains exist as mentioned earlier The capsular material is antigenic but antibodies to it fail to protect animals against infection A protective antigen distinct from the polyglutamic acid capsule is produced during the course of infection and is present in edema fluid which can be used to immunize animals against the disease Under appropriate cultural conditions as shown by Gladstone (1946) the protective antigen is formed in artificial culture medium and this finding is the basis for the preparation of the immunizing materials currently in use for the protection of man (Wright et al 1954 Strange and Belton 1954) The relation hip of the immunizing antigen to the lethal exotoxin described by Smith and Keppie and their colleagues (for review see Smith and Keppie 1955) has not been clarified precisely although they occur together in blood and tissue fluids of experimental animals and antibodies to both are protective It is possible that the immunizing antigen is a spontaneously toxoided form of the lethal toxin The recent demonstration that the lethal toxin is formed when *B anthracis* is cultivated in vitro gives promise that the complicated tangle of multiple aggressins and immunizing antigens will be unraveled (Harris Smith Smith and Keppie 1957) It is quite clear despite the present complexities that although virulent strains uniformly possess the antiphagocytic polyglutamic acid capsule it is not a part of the protective antigen

3 *Nontoxic nonantigenic surface components which inhibit phagocytosis* Two examples may be cited the hyaluronic acid capsules of streptococci of Groups A and C and the fibrin coating derived from the host which is deposited on the surface of staphylococci that form coagulase

In addition to M proteins at the cell surface which on the basis of immunologic evidence are the primary determinants of pathogenicity Group A streptococci form a second surface component hyaluronic acid that is also antiphagocytic though less active than the M proteins Hyaluronic acid is non antigenic in man and other animals because similar or identical polymerized acidic polysaccharides are distributed throughout the

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To determine precisely the part that a par ticular cell component plays in the patho genicity of a bacterial species may be very difficult In pneumococcus the situation is de ceptively simple since invasiveness can be shown by a variety of methods to depend upon a single morphologic structure the poly saccharide capsule which is antigenic More over complete protection against infection is provided by antipolysaccharide antibodies In general demonstration that antibodies to a cell fraction are protective has afforded the most secure basis for inferring that it deter mines or *influences pathogenicity In cases* such as the anthrax bacillus in which anti bodies to the polyglutamic acid capsule do not prevent infection more indirect methods must be used such as the demonstration in vitro that the capsule inhibits phagocytosis In Group A and Group C streptococci the hyaluronic acid capsules are not antigenic but appear to influence pathogenicity because it has been shown that infected animals may be afforded a degree of protection if they are injected with the enzyme hyaluronidase which digests away the hyaluronic acid cap sule in vivo

If antibodies to a cell product prevent in fection this is very strong evidence that it is a virulence factor For example, antibodies to the M proteins on the surface of Group A streptococci are protective However it is much more difficult to specify the role of other cell products such as toxins or extracellular enzymes of streptococci in the infectious proc ess because antibodies to them do not prevent disease although they may modify its course Demonstration that an isolated product is toxic such as the O hemolysin of several bac terial species cannot be taken to mean that it has significance as a determinant of patho genicity At the same time one should not infer that such products have no influence on the course of disease on the grounds that anti bodies to them do not prevent *initiation* of infection It would seem to be more reasonable to consider the M proteins as the primary *determinants of virulence and the other cell* products as secondary candidates that may come into operation after infection has been established

The species listed in Table 3 have been chosen to exemplify the multiplicity of factors that are involved in the pathogenicity of bac teria and to indicate something of their nature and mode of action Complete information is not available for any species Even in the case of pneumococcus which has been studied more extensively than any of the other species shown in Table 3 we are entirely ignorant of the factor or factors produced by it which cause death of the infected host It is abun dantly clear that the nontoxic polysaccharide capsules are of primary importance in en abling pneumococcus to be invasive and equally plain that death of the host cannot *be ascribed to a direct toxic action of the* polysaccharides

1 *Nontoxic antigenic surface components which act by inhibiting phagocytosis and anti bodies to which are protective* The polysac charide capsules of pneumococci already re ferred to are examples of this category Through inhibition of phagocytosis they per mit multiplication of bacteria in the tissues Antibodies to them promote phagocytosis and protect against infection Many other impor tant pathogens such as *Hemophilus influen ae* and *Klebsiella pneumoniae* likewise owe their chief pathogenic activity to the protection against phagocytosis afforded by antigenic polysaccharide cap ules

Proteins on the bacterial surface may act

doubt lingers as to whether or not it is adequate to exclude them entirely

Many of the substances in this category are extracellular enzymes that is to say they are produced during active growth of the bacteria in vitro as are the classic exotoxins and do not depend on autolysis for their release They are also excreted during growth of the micro organisms in the natural disease as may be determined either by their pathologic effects or because specific antibodies to them appear during the disease or in convalescence

Group A streptococci produce more extracellular toxic and enzymatic products than are recognized for any other human pathogen Of these *erythrogenic or scarlatinal toxin* is responsible for the skin rash in scarlet fever which is essentially a streptococcal infection caused by a toxin producing strain in a person who has not acquired immunity to the toxin But erythrogenic toxin has no influence upon the ability of streptococci to initiate infection so far as we are aware Its effect becomes apparent after invasion by streptococci has occurred Antibodies to the toxin which specifically neutralize its toxic action appear upon recovery The manner in which erythrogenic toxin affects the small blood vessels to produce the scarlatinal blush is unknown

Most strains of hemolytic streptococci of Group A as well as some Group C and G strains produce an extracellular enzyme *streptokinase* which brings about digestion of fibrinogen and lysis of fibrin clots The remarkable specificity of streptokinase for fibrin was first described by Tillett and Garner (1933) who termed it fibrinolysin Subsequently it was shown by Christensen and MacLeod (1945) that it is a kinase which activates an enzyme precursor plasminogen in the plasma of man and animals to form the proteolytic enzyme plasmin Plasmin has affinity for fibrin and fibrinogen both in the test tube and in vivo and it seems likely that the thin exudates characteristic of streptococcal infections are due to the action of streptokinase At any rate as soon as the antibody antistreptokinase makes its appearance during the disease in man the exudates become thick and fibrinous presumably because antibody has neutralized the kinase It is not known whether streptokinase has other pathologic effects although both it and streptococcal hyaluronidase have been invoked to explain the typically rapid extension of streptococcal cellulitis through digestion of fibrin barriers and the intercellular ground substance Antistreptokinase does not prevent infection and information is not available that demonstrates whether it modifies the disease except as indicated above

In addition to erythrogenic toxin and streptokinase Group A streptococci produce two extracellular hemolysins or cytolysins streptolysin O and streptolysin S neither of which has been shown to take part in the pathogenesis of the infection although both are very powerful cytotoxins that cause death of animals when injected with very small doses Streptolysin O is released during infection as indicated by antibody production to it Streptolysin S in all probability is also excreted by the bacteria during disease but is not so easily detectable because either it is not antigenic or else the antibody to it does not neutralize its toxic action

Streptolysin O is a protein which is readily oxidized to an inactive form by atmospheric oxygen It is related antigenically to oxygen labile hemolysins produced by pneumococcus (pneumolysin) *Cl tetani* (tetanolysin) and *Cl welchii* (θ toxin) Significantly none of the O hemolysins of these 3 species in common with streptolysin O has been shown to affect the course of natural or experimental disease

Certain strains of Group A streptococci produce an extracellular proteolytic enzyme of the papain type which is activated by KCN and sulfhydryl compounds The enzyme destroys M protein present on the surface of living cells and streptococci which produce large amounts are avirulent for mice Enhancement of virulence by mouse passage is accompanied by a diminished capacity of strains to produce the proteinase (Elliott 1945) Although production of the enzyme may affect the virulence of the bacterial cells themselves through digestion of M protein there is no evidence that its production in vivo is detrimental to the tissues of the infected host

Recently Wilson (1957) has investigated the leukotoxicity of streptococci a property first described in 1918 by Levaditi This effect which is entirely distinct from the leukocidal action of the oxygen labile hemolysin streptolysin O occurs only after the bacteria are ingested and results in death of the phagocyte Filtrates of leukotoxic streptococci do not damage the phagocytes and a protective effect of antiserum cannot be dem

animal body as constituents of the intercellular ground substance Evidence for the pathogenic role of hyaluronic acid in streptococci was obtained originally by Hirst (1941) who found that experimental infections of mice by Group A streptococci can be modified by injecting crude hyaluronidase preparations from leeches which act by depolymerizing the polysaccharide Hirst found no effect of hyaluronidase in Group A infections Subsequently Kass and Seastone (1944) using hyaluronidase prepared from bull testes reported a modifying effect on Group A infections of mice

Pathogenic strains of staphylococci produce an active substance known as *coagulase* probably an enzyme or enzyme activator which in concert with a factor present in the plasma of certain species brings about coagulation of fibrinogen Coagulase causes fibrin to be deposited on the surface of the staphylococci thereby forming a sort of capsule derived from the host which protects the microbes from phagocytosis (Smith Hale and Smith 1947) Coagulase may also play an important part in producing the characteristic walled off lesions of staphylococcal infections which may promote the disease process since the bacteria in the focal lesions are protected against the activity of the various defense mechanisms of the host Pathogenic strains of staphylococci uniformly produce coagulase and from this finding it might be inferred that coagulase is the primary determinant of pathogenicity However such an inference is unwarranted because the participation of one or another of many factor produced by pathogenic staphylococci may be more important than coagulase As shown by Rogers and Tompsett (1952) the antiphagocytic action of coagulase is feeble indeed when compared with the antiphagocytic power of virulent pneumococci, *K pneumoniae* or Group A streptococci These investigators also made the very significant observation that coagulase producing pathogenic strains of streptococci are able to survive within leukocytes which they eventually destroy whereas nonpathogenic strains fail to survive inside the phagocytes

4 *Toxic antigenic surface components (O antigens) of the enterobacteria and other gram negative bacteria* The part played by these lipopolysaccharide endotoxins in the pathogenesis of infections caused by gram negative bacteria is not clear The toxic portion now believed to be the phospholipid moiety is pyrogenic and causes a multitude of other poisonous effects mediated in part through potentiation of the action of epinephrine as shown by Zweifach and Thomas (for review see Thomas, 1954) The toxin is not antigenic, but animals repeatedly injected with it develop "tolerance which depends on cells of the reticuloendothelial system and cannot be transferred passively by the animal s serum Antibodies to the whole O antigen afford a measure of protection against experimental infection Protection appears to be due to antibodies to the polysaccharide portion which is responsible for serologic specificity The isolated polysaccharide neutralizes the opsonic effect of anti O antibody (Boivin and Delaunay 1945)

5 *Exotoxic proteins which account for the principal pathogenic properties of bacteria* This category is exemplified by the classic exotoxins produced by *C diphtheriae Cl tetani* and *Cl botulinum* Injection into animals of the purified toxins in minute amounts reproduces the significant pathologic changes in each case and antibodies directed against the toxins are protective In none of these diseases is there invasion of the living tissues of the host indeed, *Cl botulinum* should not be classed as an infectious agent The toxins of the 4 types of *Cl botulinum* are formed outside the animal body and following ingestion are absorbed through the wall of the gut

A brief description of the chemistry and the pharmacology of some important bacterial exotoxins is given below

6 *Extracellular products of bacteria that are not the principal pathogenic factors but may contribute to the disease picture* Examples which fall into this category are numerous In some instances the evidence is good for their participation in the natural disease in many there is as yet no solid evidence that they contribute to the pathologic changes In a third group such as the hyaluronidases of many bacterial species and the collagenase of *Cl welchii* although the experimental evidence would appear to rule them out as significant pathogenic factors

of iron present in the medium in which toxigenic diphtheria bacilli are growing determines the amount of toxin formed. Under the conditions of their studies the amount of toxin increased as the iron concentration was increased up to 100 micrograms of iron per liter which yielded peak toxin production. As iron was increased beyond 100 micrograms per liter toxin production declined although growth improved. At 500 micrograms Fe per liter, toxin production no longer could be demonstrated. In explanation of the seemingly paradoxical relation between iron and toxin production it should be pointed out that whereas iron is essential for growth of the organisms, no toxin is formed by the bacteria until the medium has been exhausted of iron (Mitsuhashi, Kurokawa and Kojima, 1949). At iron concentrations found optimal for toxin formation by laboratory strains no correlation has been found between toxin production of different strains isolated from patients and the severity of the disease they cause. Mueller (1941) observed that the amount of iron present in diphtheritic membrane is many times that found to be optimal for toxin formation in vitro, and that if a correlation exists between the amount of toxin and clinical severity it should be looked for under conditions where iron is in excess. Mueller tested a *gravis* strain and 3 *mitis* strains under these conditions and found that in presence of excess iron the *gravis* strain formed from 12 to 15 times as much toxin as the *mitis* strains. Therefore, it is tempting to relate differences in virulence of diphtheria bacilli to toxin production at high iron concentrations although this has not been shown to occur in vivo. Pappenheimer (1955) has reported the growth rate of a *gravis* strain to be about 3 times as fast as a *mitis* strain, the generation times being 60 and 160 minutes, respectively. If this difference in growth rate exists also in the diphtheritic membrane, it might be expected that *gravis* strains would produce more toxin in vivo. The precise relationship of iron concentration to the bacteriophage infection of *C. diphtheriae*, which must be present if toxin is to be formed (Freeman, 1951), has not been defined (for discussion see Pappenheimer, 1955).

ENHANCEMENT OF VIRULENCE

Enhancement of bacterial virulence is readily demonstrable experimentally upon animal passage of bacterial strains. For example, most Group A streptococcal strains and some pneumococci on primary isolation from man are of low virulence for mice. By repeated mouse passage, virulence of pneumococcus may be increased from an LD_{50} of 100,000 bacteria or more to a degree where less than 10 cocci uniformly cause fatal infection. Enhancement of virulence by animal passage results from selection of virulent mutants present in the original heterogeneous culture or that have arisen upon multiplication in the animal body.

It is commonly believed that during the course of epidemic disease in man the virulence of the infecting agent increases. While this seems to be reasonable and may be true, there is no objective evidence to support it. The many factors involved in the spread of an epidemic disease make it almost impossible to establish that virulence has increased. Perhaps the greatest difficulty in analysis lies in the fact that virulence for laboratory animals is rarely a measure of virulence for man, and since virulence for man cannot be determined under accurately controlled conditions, any inferences must be based upon epidemiologic analysis. Such analysis has not brought forth proof of increasing bacterial or viral virulence during the course of a human epidemic. To the contrary, it would appear more likely that a highly virulent and communicable mutant was selected through one means or another *before* the epidemic began. If this were not the case it is difficult to visualize how an epidemic could start.

A possible exception to the remarks in the preceding paragraph is the appearance of drug resistant mutants of bacteria under circumstances where large numbers of infected persons are treated over an extended period or where the drugs are used in low dosage for prophylaxis. Under such circumstances sulfonamide resistant gonococci and Group A streptococci have appeared and have partially replaced the sulfonamide susceptible strains previously encountered as the causes of epidemic disease of man. There is no evidence that the resistant mutants are more virulent for *normal* man than susceptible strains. However, the additional attribute of drug fastness which enables resistant strains to cause disease in persons treated with sulfonamides can perhaps be termed an enhancement of virulence since sulfonamide sensitive strains are not able to cause disease under the same circumstances.

onstrated It appears that the leukotoxin is able to damage cells only if it is liberated within them and to this end the streptococci serve as Trojan horses Leukotoxin is pro duced by many but not all strains of Group A streptococci and its formation is associated especially with certain M types such as Type 12 The formation of leukotoxin is not cor related with mouse virulence Bernheimer, Lazarides and Wilson (1957) have docu mented an interesting association between leukotoxicity and the production by certain strains of an enzyme streptococcal DPNase which destroys the coenzyme diphosphopyri dine nucleotide The identity of leukotoxin and DPNase has not been proved As in the case of a number of other streptococcal extra cellular products no correlation has been shown between their activity and virulence but as Wilson (1957) points out it is possible that in the contest between the invading bac teria and the cells of the host the leukotoxin might at times determine whether infection will be established or not The same might be said of other activities of Group A strepto cocci

Cl welchii the most frequent cause of gas gangrene in common with other clostridia produces a powerful proteolytic enzyme, *col lagenase* which is capable of disintegrating muscle of laboratory animals in vivo by de composing the reticular framework Pulping of affected muscles is a characteristic of clostridial myositis However it is by no means clear that collagenase contributes in a significant way to the pathogenesis of infec tion by *Cl welchii* Indeed the weight of evidence is against it since the specific anti body anticollagenase neither prevents infec tion nor appears to modify its course Simi larly antibodies to *Cl welchii hyaluronidase* do not prevent infection nor do they mitigate the progress of the lesion Protection against infection depends upon antibodies to the *α toxin* a *lecithinase* which hydrolyzes lecithin to phosphoryl choline and a diglyceride The lecithinase causes rapid lysis of erythrocytes and necrosis of other cells and can account for most of the severe local effects following infection by *Cl welchii* However it is not proved that death from gas gangrene is caused by α toxin absorbed from the involved muscle and disseminated throughout the body Pro found shock is seen in severe and fatal cases but free toxin is not demonstrable in the circulation nor does intravascular hemolysis occur except in cases of septic abortion in which in contrast with clostridial myositis, the clostridia themselves are present in the blood

VARIATIONS IN VIRULENCE ASSO CIATED WITH THE PRODUCTION OF DIFFERENT AMOUNTS OF A PATHOGENIC FACTOR

Variations in virulence between strains of the same species or type may depend not only upon the presence or the absence of a factor known to be concerned in pathogenicity but also upon the amount produced For ex ample, in pneumococcus loss of capsulation through S ⟶ R mutation results in loss of virulence Mutants of intermediate virulence are commonly encountered and in strains of 3 different types it has been shown that viru lence for mice is correlated with the amount of capsular polysaccharide produced in vitro (MacLeod and Krauss 1950) Studies by Wood (for summary see Wood 1951 52) ex plain in part the influence of different amounts of polysaccharide on virulence since he has shown that pneumococci that possess large capsules are phagocyted with great difficulty Moreover if a strain produces large amounts of polysaccharide a lag in opsonization re sults because the host must produce larger amounts of specific antibody to combine with the excess of free polysaccharide in the tissues and body fluids as well as that present on the surface of the bacteria themselves The delay in opsonization permits more extensive multi plication Differences in capsule size corre sponding to the amount of polysaccharide formed in vitro can be demonstrated in vivo In *Klebsiella pneumoniae* it has been found also that the amount of capsular polysac charide and its rate of production bear a relationship to virulence (Ehrenworth and Baer 1956)

A similar quantitative relationship may ex ist in diphtheria bacilli in which the exotoxin is the chief determinant of pathogenicity The simplest explanation of why *gravis* strains in general cause a more severe type of diphtheria than *mitis* strains would be that they are able to produce more toxin in vivo Pappenheimer and Johnston (1936) showed that the amount

species show great variation in susceptibility to bacterial toxins (Metchnikoff 1905) Man horse and guinea pig are extremely susceptible to tetanus botulinus and diphtheria toxin The mouse and the rat are only about 1/1000th as sensitive as the guinea pig to diphtheria toxin The dog while very resistant to botulinus and tetanus toxins, is highly sensitive to diphtheria toxin Rabbits are approximately 10 000 times as susceptible to the killing action of shiga neurotoxin as guinea pigs

TABLE 4 AMINO ACID COMPOSITION OF TETANUS AND BOTULINUS TOXINS AND OF RABBIT α GLOBULIN (ANTIBODY TO PNEUMOCOCCAL POLYSACCHARIDES)

	TETANUS TOXIN[1]	BOTULINUS TOXIN[2] TYPE A	RABBIT[3] GLOBULIN
	per cent	per cent	per cent
Nitrogen	15 7	16 3	16 0
Sulfur	1 04	0 44	1 32
Cystine	—	0 53	2 25
Cysteine	—	0 27	—
Methionine	1 78	1 06	1 40
Tyrosine	—	13 5	6 62
Tryptophane	0 91	1 86	2 70
Phenylalanine	4 91	1 1	5 49
Arginine	3 36	4 62	5 04
Histidine	1 15	1 03	1 53
Lysine	10 0	7 4	6 46
Aspartic acid	15 3	20 26	9 67
Glutamic acid	10 3	15 57	11 8
Isoleucine	9 36	11 94	4 39
Leucine	8 23	10 30	7 90
Threonine	5 13	8 49	13 22
Valine	5 39	5 29	10 22
Glycine	3 34	1 38	5 72
Alanine	—	5 92	5 68
Serine	—	4 36	10 92
Proline	—	2 60	8 56
Sedimentation constant	4 5S	17 3S	6 3S
Molecular weight	67 000	900 000	160 000

[1] Dunn M S Camien M N and Pillemer L Arch Biochem 1949 2 374 3 6

Buehler H J Schantz E J and Lamanna C J Biol Chem 1947 169 295 30

[3] McFadden M L and Smith E L J Biol Chem 1955 14 185 196

Smith F L McFadden M L Stockell A and Buettner Janusch V J Biol Chem 1955 14 197 207

Cold blooded animals are completely resistant to large doses of tetanus botulinus and diphtheria toxins with the interesting exception of the frog and certain lizards Injection of tetanus toxin into these amphibia has no effect at low temperatures but symptoms of tetanus appear when the creatures are kept at temperatures above 20 C

On the basis of the amount of purified toxin required to produce death (LD_{50}) in a susceptible species such as the guinea pig tetanus and botulinus toxin the most poisonous substances known are about 400 times as toxic as diphtheria toxin The reason for the difference may lie in the fact that tetanus and botulinus toxins have selective affinity and toxicity for limited portions of the nervous system whereas diphtheria toxin is a general poison that is taken up by and affects any cells it comes in contact with

Chemical analysis of bacterial toxins has not provided an explanation for their extraordinary toxicity Reasonably complete information on amino acid composition of tetanus and type A botulinus toxin is available as shown in Table 4 in which for the sake of comparison data are presented for a well characterized protein rabbit γ globulin antibody to pneumococcal polysaccharides No unusual chemical groupings that can account for toxicity have been discovered although it should be emphasized that mapping of amino acid sequences and manner of coiling of the polypeptide chains has not been carried out Dunn et al (1949) have noted that in tetanus and type A botulinus toxins the ratios of aspartic acid to glutamic acid and of isoleucine to leucine are reversed as compared with most other proteins that have been studied Toxicity appears to depend on the spatial configuration of amino acids and peptide chains within the intact protein molecule and any procedure which alters the protein in any way results in loss of toxicity

TOXOID FORMATION

A variety of agents which react with bacterial toxins cause irreversible loss of toxicity without loss of antigenicity or of power to combine with antitoxin All of these reagents apparently attack free amino groups they include iodine ketene (an acetylating agent) and diazonium salts However the most com

Virulence is also said to be increased by certain artificial methods such as the injection of bacteria suspended in mucin Enhancement of virulence by such methods should be regarded as more apparent than real Meningococci typhoid bacilli staphylococci and some other bacterial species are quite avirulent for mice and to cause death these animals must be injected in relatively enormous doses, often 0 1 to 1 ml of broth culture The number of living organisms required to bring about a fatal 'infection does not differ markedly from the number of heat killed bacteria or their toxic products which cause death on injection If living meningococci or typhoid bacilli are suspended in a viscous protective medium such as mucin fatal infections result in mice after intraperitoneal injection of only a few bacteria The small inoculum protected by a surrounding coating of mucin presumably multiplies until sufficient bacteria are present to constitute a lethal toxic dose It may be added that studies of virulence in laboratory animals using bacteria suspended in mucin have provided little insight into the mechanisms involved in virulence and moreover have no relationship to virulence for man

Just as the virulence of bacteria may be increased by selection through repeated animal passage so it may often decrease by continued cultivation on artificial media The process again appears to be one of selection in this case of *less* virulent mutants Many examples of such loss of virulence on laboratory media following isolation might be given of which only one will be discussed here *Cl septicum* a strict anaerobe not infrequently associated with wound infections in man produces a potent exotoxin when cultivated in meat broth following isolation from the infected tissue If the organisms are transferred a few times in a chemically defined medium which supports luxuriant growth their capacity to produce this toxin is lost and at the same time the strain loses its virulence (Bernheimer 1944) If such cultures are plated on blood agar, the colonies are found to be rough in form as contrasted with the smooth colonies formed by freshly isolated virulent toxin producing strains It seems probable that the chemically defined medium is lacking in some growth factor present in animal tissue which is required by the smooth virulent organisms but is not required by the rough mutants The factor is apparently present in meat broth since the change from smooth to rough occurs more slowly on transfer in this medium

CHEMISTRY AND PHARMACOLOGY OF BACTERIAL TOXINS

Classic Exotoxins of Gram Positive Bacteria

Several examples of diffusible toxins produced by gram positive bacteria have been referred to already These substances have been called exotoxins because they are found in filtrates of growing organisms exhibiting no visible evidence of autolysis or else are found to increase in amount parallel with growth In some instances such as *Cl botulinum* and *Cl tetani* the yield of toxin is increased upon autolysis Exotoxins are characteristic of gram positive bacteria and are produced by many pathogenic species Gram negative bacteria, on the other hand, produce lipopolysaccharide endotoxins which form a part of the O or somatic antigen and are intimately *associated with the structural integrity of the* cells They are not liberated into the medium unless autolysis has occurred An exception among gram negative bacteria is the type species *Shigella dysenteriae* that produces a very potent protein exotoxin the so called shiga neurotoxin distinct from the endotoxin Antibodies to the neurotoxin neutralize its toxicity but do not protect against infection It is not established that shiga neurotoxin contributes to the pathogenic properties of *Sh dysenteriae* under natural conditions

The toxins produced by *C diphtheriae Cl tetani* and *Cl botulinum* type A have been isolated in highly purified form as heat labile proteins Indeed the toxins of tetanus (Pillemer et al 1946) and type A botulinus have been obtained in crystalline form (Abrams et al 1946 Lamanna et al 1946) These proteins in common with shiga neurotoxin are among the most powerful poisons known It has been estimated that one milligram of crystalline tetanus or botulinus toxin is sufficient to kill 1 000 tons of guinea pig The minimal lethal dose of botulinus toxin for the mouse is only 20 000 000 molecules Different animal

species show great variation in susceptibility to bacterial toxins (Metchnikoff 1905) Man horse and guinea pig are extremely susceptible to tetanus botulinus and diphtheria toxin The mouse and the rat are only about 1/1000th as sensitive as the guinea pig to diphtheria toxin The dog while very resistant to botulinus and tetanus toxins is highly sensitive to diphtheria toxin Rabbits are approximately 10 000 times as susceptible to the killing action of shiga neurotoxin as guinea pigs

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Phenylalanine	4 91	1 17	5 49
Arginine	3 36	4 62	5 04
Histidine	1 15	1 03	1 53
Lysine	10 0	7 74	6 46
Aspartic acid	15 3	20 26	9 67
Glutamic acid	10 3	15 57	11 8
Isoleucine	9 36	11 94	4 39
Leucine	8 23	10 30	7 90
Threonine	5 13	8 49	13 22
Valine	5 39	5 29	10 22
Glycine	3 34	1 38	5 72
Alanine	—	3 92	5 68
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TOXOID FORMATION

A variety of agents which react with bacterial toxins cause irreversible loss of toxicity without loss of antigenicity or of power to combine with antitoxin All of these reagents apparently attack free amino groups they include iodine ketene (an acetylating agent) and diazonium salts However, the most com

monly used reagent is dilute formaldehyde It was discovered by Glenny and Hopkins and by Ramon that crude diphtheria toxin could be completely detoxified by treatment with dilute formalin (0 4 0 5%) at slightly alkaline pH The reaction is usually complete after 3 or 4 weeks at 37° C The detoxified product termed *anatoxine* by Ramon and *toxoid* by Glenny and Hopkins (1923), retains its immunologic specificity and antigenic properties Tetanus and botulinus toxoids may be prepared in a similar manner by treatment of the corresponding toxins with formalin Diphtheria and tetanus toxoid are used on a large scale for active immunization of man against diphtheria and tetanus

The change from toxin to toxoid is a property common to most bacterial toxins and is a process which can occur spontaneously to a certain extent even at low temperatures This spontaneous detoxification without parallel loss in immunologic combining power was first observed by Paul Ehrlich in 1903 who was in fact the first to use the term ' toxoid

Pharmacologic Action of Diphtheria Tetanus and Botulinus Toxins and Lecithinase of Cl welchii

The pharmacologic action of each of these toxins is different Diphtheria toxin causes damage to almost all types of cell in the susceptible animal whereas the action of botulinus toxin is restricted to cholinergic nerve endings in peripheral autonomic and somatic fibers and tetanus toxin appears to affect only the motor neurons in the cerebrospinal axis As noted above the selectivity of action of tetanus and botulinus toxins can well account for their extraordinary toxic effect as measured by their LD_{50} in susceptible animals The α toxin of *Cl welchii* is a lecithinase

Pappenheimer and his associates (for review see Pappenheimer 1955) have accumulated suggestive evidence that the toxicity of diphtheria toxin is due to its interference with the cytochrome system of the cells of susceptible animals Toxin is formed by diphtheria bacillus only in the presence of oxygen and a supply of iron less than is necessary for optimal growth In studies of the Park Williams 8 strain which is used in most laboratories for toxin production it has been shown that bacteria harvested from cultures in which an amount of iron optimal for toxin formation is present have a very low iron content, and that all iron containing respiratory enzymes are reduced in amount This is particularly striking in the case of cytochrome b_1 the principle respiratory pigment to which 9/10ths of the hemin iron is bound As toxin is produced by iron depleted cells coproporphyrin III is also excreted into the medium The ratio of toxin to porphyrin is 1 4, the same as is present in iron containing respiratory enzymes This observation suggested to Pappenheimer that toxin may be related to the protein portion of diphtherial cytochrome b_1, and that toxin may cause injury to the cells of susceptible animals by interfering with cytochrome b a respiratory pigment which is similar to diphtherial cytochrome b_1 and like it is concerned in succinate oxidation This ingenious scheme to account for the toxicity of diphtheria toxin has not yet been proved but strong indirect evidence for it has been presented

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In contrast with the action of diphtheria toxin and lecithinase of *Cl welchii* botulinus and tetanus toxins act only on the nervous system (for review see Wright 1955) The

parallelism between the sites of action of acetylcholine and botulinus toxin made it appear probable that botulinus toxin has an anticholinergic action and it has been shown that the toxin acts very widely on all parts of the peripheral nervous system that are cholinergic whether they are preganglionic or postganglionic components of the autonomic system or are the motor innervation of skeletal muscles The studies of Burgen Dickens and Zatman (1949) demonstrated that botulinus toxin depresses the release of acetylcholine and the pharmacologic effects can be explained best on this basis

Whereas botulinus toxin acts on peripheral nerves the action of tetanus toxin is in the cerebrospinal axis only and there is no clear evidence that it has any action on peripheral nerves From its site of introduction tetanus toxin travels to the central nervous system along peripheral nerves Early studies suggested travel within the axon itself but the later work of Baylis and his colleagues (1952) indicates strongly that the toxin moves centripetally in the interneuronal tissue spaces propelled by the pressure from contracting muscles and enters the interstitial tissue fluid of the central nervous system at the point of emergence of the peripheral nerve trunks from the spinal cord Its effect is on motor neurons exclusively Recently Ambache and his associates have demonstrated in local *tetanus intoxication of the eye that the formation of acetylcholine is greatly reduced There is evidence therefore* that both botulinus and tetanus toxin act through interference with *cholinergic mechanisms* and that the mode of action in each case may be similar The *great difference in the action of the toxins is* that tetanus acts centrally in the cerebrospinal axis whereas botulinus toxin affects only cholinergic nerve endings in peripheral somatic and autonomic fibers The degeneration of anterior horn cells observed in tetanus intoxication is believed to be a secondary manifestation and is not the cause of the characteristic symptomatology

THE RELATION OF HYPERSENSITIVITY TO DISEASE PROCESSES

Although hypersensitivity to components of the bacterial cell undoubtedly plays a considerable part in the type and the progress of the lesions in many infectious diseases especially those of chronic nature little specific information is available except in the case of tuberculosis In tuberculosis in guinea pigs the effect of hypersensitivity to tuberculoproteins which in themselves are nontoxic can be demonstrated readily Normal guinea pigs injected with as much as 2 ml of Koch s Old Tuberculin do not suffer any obvious ill effects On the other hand if a tuberculous guinea pig during the 8th or the 10th week of infection is injected with as little as 0 01 cc of tuberculin death may occur within a few hours An intense inflammatory reaction occurs at the site of injection and throughout the body wherever tubercles are present

The generalized tuberculin reaction in the guinea pig which ends in death illustrates the ill effects of the allergic reaction in its most severe form Less spectacular but nonetheless damaging effects due to the hypersensitive state that uniformly exists and the presence in the lesions of tubercle bacilli and their products almost certainly occur in the course of the tuberculous process in man and animals It seems likely that the destructiveness of the tuberculous lesions in the adult type of tuberculosis with extensive *caseation* and fibrosis is due to hypersensitivity

In other *chronic bacterial infections* for example brucellosis hypersensitivity to the bacteria and their products likewise may have an important influence on the character and the persistence of the lesions However this has not been clearly defined Up to the present time there is no good evidence that the hypersensitive state is part of the host s defense against infection

THE COMMUNICABILITY OF BACTERIA

A great deal more is known of the factors influencing the pathogenicity of micro-organisms than of the properties they must possess in order to be communicable from host to host under natural conditions Pathogenicity is more susceptible to experimental study because of its end result—disease—whereas *or*ganisms may be transmitted from host to host without any detectable pathologic alteration *For this reason* the study of properties concerned in communicability of micro-organisms

monly used reagent is dilute formaldehyde It was discovered by Glenny and Hopkins and by Ramon that crude diphtheria toxin could be completely detoxified by treatment with dilute formalin (0 4 0 5%) at slightly alkaline pH The reaction is usually complete after 3 or 4 weeks at 37° C The detoxified product, termed *anatoxine* by Ramon and *toxoid* by Glenny and Hopkins (1923), retains its immunologic specificity and antigenic properties Tetanus and botulinus toxoids may be prepared in a similar manner by treatment of the corresponding toxins with formalin Diphtheria and tetanus toxoid are used on a large scale for active immunization of man against diphtheria and tetanus

The change from toxin to toxoid is a property common to most bacterial toxins and is a process which can occur spontaneously to a certain extent even at low temperatures This spontaneous detoxification without parallel loss in immunologic combining power was first observed by Paul Ehrlich in 1903 who was in fact the first to use the term toxoid

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variants alone are involved presumably be cause only these variants would have sur vived in the original host The size of the inoculum here should play only a small part since most of the bacterial population would consist of communicable variants However in transmission from one species of animal to another it is probable that the size of the inoculum is of considerable significance be cause the conditions necessary for survival in the new host are likely to be different from those in the original host if the inoculum is large there is more chance of communicable mutants being present than if it is small

Chance In transmission from one host to another of the same species chance may de termine largely whether or not the new host becomes a carrier although even in indi viduals of the same species there may be differences which influence the capacity to be come carriers By chance it meant simply the accident of coming in contact with a bacterial species or strain The operation of chance is well illustrated in the communicability for man of pneumococci (Hodges and MacLeod 1946) From 40 to 70 per cent of normal adult humans carry one or more of the many sero logic types of pneumococci in the pharynx at any given time some persons carrying as many as 5 distinct types all at once The ratios of carriers of 2 types to 1 type 3 types to 2 types etc as shown in Table 5 are approximately the same indicating that in the main chance has determined how many types of these communicable organisms are carried by an individual

However further analysis of pneumococcal carriers shows that host factors also operate in determining whether or not an individual will become a carrier of a particular pneumo coccal type (MacLeod Hodges Heidelberger and Bernhard 1945) In a population of which half the members were immunized against pneumococcus Types 1 2 5 and 7 it was found that significantly fewer of the im munized men were carriers of these types as compared with the nonimmunes It is appar ent therefore that pneumococci are not as communicable to immune persons as to non immunes Analogous data are not available for other bacteria although a similar state of affairs probably exists for diphtheria bacilli also In this case immunization of approxi

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mately half of the susceptible population affords a very considerable measure of pro tection to the nonimmunized portion as was found true also for pneumococcal infection in a partially immunized population The chain of transmission from one susceptible individual to another appears to be broken by the interposition of immune individuals who are less able to act as carriers In other words the immune status of the individual in the case of pneumococci and probably diphtheria bacilli exerts a powerful influence on communicability

Survival Capacity in Immune Subjects Whereas with certain bacteria the immune individual is less likely to become or remain a carrier than the nonimmune the capacity of many bacterial species to survive in im mune subjects is an important contributing factor in communicability An obvious ex ample is the persistence of typhoid bacilli in the biliary tracts of a proportion of those who have recovered from the disease whence the organisms are discharged into the intes tinal canal and through subsequent fecal contamination of food or water become trans mitted to susceptible individuals If the reser voir of infectious agents consisted only of persons actually sick with infection the con trol of infectious disease by quarantine of the sick for example would be a relatively simple procedure However since perfectly well individuals either recovered from infec tion or who at no time have shown clinical evidence of disease may harbor the fully virulent pathogen it is apparent that for

has lagged far behind knowledge of factors which influence virulence

Although communicability must be pos sessed by naturally pathogenic species, it is not confined to virulent micro organisms The bacteria which comprise the normal flora of the skin the oropharynx, the alimentary tract and the external genitalia of healthy persons are by definition communicable even though they rarely cause *disease in normal persons* Indeed, certain of the micro organisms form ing the normal flora of the alimentary canal may be essential for health through the syn thesis of vitamins or the partial digestion of nutrients for which the host does not possess enzymes For example much of the vitamin K requirement of man and animals appears to be supplied by intestinal bacteria which are able to synthesize it In ruminants bacteria in the stomachs play a very important part in the digestion of food

In contrast with micro organisms that are communicable but nonvirulent are those that are virulent when introduced into the animal body but are not communicable from experi mentally infected animals to normal cage mates For example pneumococci are virulent when introduced experimentally into mice by almost any route One or two cocci of the most virulent strains will cause the death of mice following intraperitoneal injection but normal mice kept in the same cage remain well

Because communicability and virulence are not necessarily interdependent it would ap pear that epidemic strains of a bacterial *species are those in which natural selection* has operated to permit the survival of vari ants which are concurrently highly communi cable and virulent

The site of the lesions in the infected host has an important influence on communicabil ity and this may have a bearing on the non communicability of pneumococcal infections in mice where the disease does not involve the respiratory tract primarily even though the bacteria are placed directly within the trachea but instead produces a rapidly fatal bacteremic infection If the disease were local ized in the lungs and mice had the capacity to cough and spit pneumococcal infection might occur as an epizootic disease in this species

The influence of the site of the lesion on communicability is illustrated by plague in man which occurs in two well recognized clin ical forms called "bubonic' and pneumonic plague The bubonic form is spread from rats to man through the bite of the rat flea Buboes form in areas adjacent to the point where the organisms are introduced through the bite of the flea, and although invasion of the blood *stream commonly occurs* secondary cases of plague do not arise because the bubonic form is essentially a closed infection On the other hand pneumonic plague caused by the same bacterial species *Pasteurella pestis* is con tagious to those in proximity to the sufferer who expels enormous numbers of plague bacilli during coughing and in the copious discharges from the respiratory tract Sec ondary cases of pneumonic plague occur under certain undefined environmental circum stances because the open nature of the pul monary lesions permits dissemination of the bacilli

A less exotic illustration of the influence of the site of the lesions on the communicabil ity of bacteria is afforded by the hemolytic streptococcus The expression dangerous car rier has come into current usage to indicate infected individuals from whom secondary cases arise commonly Several independent investigations have shown that patients with purulent lesions discharging on the body sur face as for example otitis media cervical adenitis and wound infections are more likely to transmit infection than patients with un complicated streptococcal pharyngitis The *reason for this difference in communicability* from different lesions caused by the same streptococcal strains may be because purulent lesions discharge more streptococci into the environment contaminating heavily every thing in contact with the patient whereas from patients with uncomplicated pharyngitis fewer organisms are discharged into the en vironment therefore there is less chance of secondary cases arising

Size of the Inoculum In the case of viru lence of bacteria the size of the inoculum is of great significance The same may also be true of communicability though this has not been demonstrated When a micro organism is transmitted from one individual of a species to another of the same species communicable

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However further analysis of pneumococcal carriers shows that host factors also operate in determining whether or not an individual will become a carrier of a particular pneumococcal type (MacLeod Hodges Heidelberger and Bernhard 1945) In a population of which half the members were immunized against pneumococcus Types 1 2 5 and 7 it was found that significantly fewer of the immunized men were carriers of these types as compared with the nonimmunes It is apparent therefore that pneumococci are not as communicable to immune persons as to nonimmunes Analogous data are not available for other bacteria although a similar state of affairs probably exists for diphtheria bacilli also In this case immunization of approximately half of the susceptible population affords a very considerable measure of protection to the nonimmunized portion as was found true also for pneumococcal infection in a partially immunized population The chain of transmission from one susceptible individual to another appears to be broken by the interposition of immune individuals who are less able to act as carriers In other words the immune status of the individual in the case of pneumococci and probably diphtheria bacilli exerts a powerful influence on communicability

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most infectious diseases quarantine of the sick is not an effective control procedure

The development of a carrier state in normal individuals who at no time have shown evidence of disease is of more significance in many instances than the persistence of the agent in recovered patients This is true in the case of meningococci for example where the number of normal persons who carry the micro organism exceeds by far those who develop meningococcal infection In this instance the person who never has been ill is of greater importance in the transmission of the micro organisms than those recovered from infection With the typhoid bacillus, on the other hand the individual who has recovered from the disease is the primary source for the maintenance and the dissemination of the bacteria Therefore it is apparent that no general rule can be laid down concerning the circumstances under which pathogens can best survive in the body With different micro organisms affecting different areas of the body the conditions of parasitism vary

The ability to survive outside of the animal body in which disease is produced or in which they can be carried intermittently may have considerable significance in the communicability of many bacteria The most striking illustrations of organisms having the ability to survive outside the animal body are the spore forming pathogens Anthrax spores may survive in pasture land for as long as 12 years as described by Pasteur in 1881 and animals feeding on it may be infected The spores of the tetanus bacillus and the anaerobic bacteria associated with gas gangrene which find their way to the soil especially through the feces of man and animals survive there for long periods of time and upon introduction into wounds may germinate and cause disease In the case of organisms existing only in a vegetative phase the capacity to survive outside of the animal body is not nearly so great as for spore formers although if vegetative forms are dried rapidly they may remain viable for long periods of time under natural climatic conditions

Numerous studies of the environment have been made in an attempt to explain the epidemiology of hemolytic streptococcal infections of the respiratory tract In places where the disease is endemic living streptococci can be isolated from clothing bedding and other articles as well as from dust and from the air itself The general contamination of the environment was long considered as being of great significance in the spread of streptococcal disease and based on this circumstantial evidence elaborate procedures were set up in military establishments to reduce it Oiling of floors to hold down dust borne streptococci had no influence on the incidence of disease nor did sterilization of air by glycol aerosols or ultraviolet light The studies of Rammelkamp Wannamaker Perry and their colleagues (for review see Rammelkamp 1955 56) have explained why such procedures are ineffective They have presented strong evidence that transmission is direct from man to man and does not appear to occur commonly at a distance through the air or because of dried streptococci present in the dust or on fomites Direct contact with fresh moist secretions of the infected person appears to be the important means of transmission Indeed these investigators have demonstrated that living but dried Group A streptococci present in floor dust are not infectious for man even when instilled into the nasopharynx in large numbers It would appear likely that they are taken up and destroyed by phagocytic cells during the prolonged lag phase before growth can be initiated from the dried state

Likewise pneumococci have been isolated from the environment in places where pneumococcal pneumonia was epidemic and it has been assumed that this environmental contamination is of importance in spread Similarly in the case of epidemic staphylococcal infections in hospitals which constitute such a serious problem at the present time air borne spread is considered as being of great importance although no solid evidence is available to prove it

Unless it is shown otherwise it would appear more reasonable to consider that when bacteria in the dried state are present as general contaminants of the environment this may have no more significance than as a reflection of the fact that disease caused by them is taking place in that environment It should not be assumed that these dried environmental deposits have a significant part to play in spread

Survival in water has an important bear

ing on communicability of enteric pathogens such as those causing typhoid or paratyphoid fevers dysentery and cholera all of which may be water borne diseases though not trans mitted in this manner exclusively

The ability to survive and multiply in an intermediate host or vector is of prime sig nificance with some micro-organisms of which plague is a good example The plague bacillus causes natural disease in many species of rodents in different parts of the world and is transmitted to man through the bite of various species of fleas which are ectopara sites of the rodents The bacilli are able to multiply and cause disease in the upper por tion of the alimentary tract of the fleas which derive their infection from the rodent reser voir The ability of plague bacilli to infect fleas is thus a crucial factor in their com municability *Pasteurella tularense* an organ ism related to the plague bacillus and the cause of tularemia has its reservoir also in various rodent species and may be carried by arthropods such as ticks and deer flies which feed on the rodents Although in the United States the disease in man is most often ac quired during the skinning and the dressing of wild rabbits it is also possible for trans mission to occur through the bite of the arthro pod vectors

Flies (*Musca domestica*) have long been suspected as being of importance in the trans mission of typhoid fever In this instance however the insect occupies a relatively passive role in that the fly is not infected by the typhoid bacillus nor does it become a permanent carrier of the micro organisms The feet of the fly and other parts of its body surface become contaminated during contact with feces and then the organisms may be transferred mechanically to human food

From this discussion it can be seen that certain of the general properties necessary for communicability of various bacterial species can be defined However as noted earlier little information of a specific nature is avail able With pathogenic species the communi cable organisms carried in the body usually pos ess the cell structures known to be asso ciated with virulence though this is not uni formly true Pneumococci isolated from normal humans are in the encapsulated that is po tentially pathogenic state and the nonencap sulated rough variants are not found On the other hand hemolytic streptococci isolated from carriers not infrequently have lost the M protein which is known to be concerned in their virulence Therefore these organisms must possess a component or components other than the important M protein which prevent their destruction by the body It can be argued that such avirulent varients lacking M protein may have no pathogenic signifi cance However upon transmission to a non immune host it is entirely possible that they may regain the capacity to produce disea e through selection of virulent M producing mutants

Part 2

DEFENSE MECHANISMS OF THE HOST

In Part 1 of this chapter the point of view has been taken that the host seeks to rid itself of parasitic micro organisms whether they are commensals that are constantly present in all members of the host species or belong to microbial species that usually are associ ated with disease To achieve homeostasis in the face of the very numerous parasitic microbial pecies the host possesses a set of defenses whose basic action is to eliminate or at least segregate foreign material whether it be living or dead organic or inorganic toxic or nontoxic The fundamental mechanism ap pears to be ingestion and destruction by phagocytic cells both fixed and wandering This primordial function is greatly enhanced by the antibody forming system since specific antibodies are able not only to combine with and detoxify many noxious materials but also upon combination with microbial cells or their products cau e them to be ingested by phago cytes at a much faster rate than when anti body is absent In addition to the phagocyte antibody complex there is a variety of other antimicrobial agencies such as the bactericidal and bacteriolytic complement system which functions in association with antibodies or properdin and the bactericidal enzyme lyso zyme which is widely distributed throughout the tissues and in various secretions Further

more nonspecific reactions such as inflammation may exert a most important influence both on implantation of a parasite and the outcome of infection

Defense Mechanisms at the Portal of Entry

The internal tissues and organs are separated from the external environment by the skin and by the mucous membranes lining the alimentary the respiratory and the genital tracts These surface membranes form a protective barrier against infection both in a mechanical way and because they possess special mechanisms which tend to inhibit destroy or remove parasites that come in contact with them Their antimicrobial capabilities are intimately related to the general defense mechanisms and their activities are discussed separately here solely for the sake of convenience

The intact *skin* of the body has the capacity to destroy most bacteria that contaminate it and few agents are able to penetrate it It has long been held that bacteria of the genus *Brucella* and *Pasteurella tularense* which commonly cause laboratory infections can penetrate the intact skin and cause generalized infection This has not been proved and it seems equally probable that these microorganisms gain entrance through skin breaks or else through the conjunctiva the respiratory mucous membranes or reach the alveoli directly through inspired air Once the integrity of the skin has been broken whether by wounding maceration or because of a physiologic abnormality infection is set up readily Of all the microbial species pathogenic for man staphylococcus is best adapted to cause infection of the skin but even in this infection obvious physiologic abnormalities are often present as predecessors For example in acne vulgaris of adolescence plugging and damage of sebaceous glands and ducts by inspissated secretions occur and then staphylococcal infections can be set up in them The antibacterial mechanisms of the skin are poorly defined but it seems reasonable to conclude that fatty acids in the secretions of the sebaceous glands are important both as antifungal and antibacterial agencies and possibly also the acid salty output of the sweat glands The carbohydrate-splitting antibacterial enzyme lysozyme is present in the skin and presumably is part of its antibacterial defenses

The *nose* and the *nasopharynx* have specialized abilities to inhibit entrance of foreign particles into the deeper portions of the respiratory tract From the outer half centimeter or so of the nasal passages which is covered by skin staphylococci can commonly be isolated as is true of the skin of other areas of the body, but the remainder of the nasal passages contain very few bacteria or are found to be sterile The hairs at the nasal orifices function by preventing entrance of large particles Particles that pass the external nares become entrapped in the mucus which coats the mucous membrane of the nose and are swept backward to the nasopharynx by the cilia of the epithelial cells, to be disposed of by swallowing However these mechanical properties by themselves are insufficient and more subtle agencies are responsible for most of the antimicrobial activities

Two important humoral factors, lysozyme and specific antibody have been found in nasal mucus Lysozyme described by Fleming and Allison in 1922 is an enzyme that hydrolyzes a structural polysaccharide component of the cell wall of certain nonpathogenic and pathogenic bacterial species resulting in death and lysis It is present in high concentration in tears and nasal mucus and in lesser amount in the saliva The enzyme occurs in most human secretions and tissues including the leukocytes

Francis described the presence of specific antibody to influenza virus in nasal mucus of normal persons (for review see Francis 1941 42) The concentration in mucus was found to be about one tenth that of the antibody present in the person s blood Little attention has been paid to this observation although it seems not unlikely that specific antibody on the surface of the mucous membranes is an important aspect of their defenses against superficial infections such as influenza and other viral infections of the upper respiratory passages Indirect evidence for the role of antibody is provided by the finding that men immunized against various pneumococcal types have a much reduced capacity to act as nasopharyngeal carriers of the same types as compared with normal unimmunized men in the same environment (MacLeod et al 1945) Presumably specific antibody present on the surface of the mucous membrane combines

with the pneumococci and renders them susceptible to ingestion by the free and fixed phagocytes that are present in and on the surface of the membranes It should be recalled also that the antibody level to diphtheria toxin in the blood serves as a measure of the ability to prevent pharyngeal infection by diphtheria bacilli

In response to irritating stimuli there is an outpouring of phagocytic cells and serum on the surface of the mucous membranes which might be expected to enhance the ability to dispose of foreign material although it is by no means clear that this is the usual result, as indicated below If the parasite or inert particle penetrates the mucous membrane whether within phagocytic cells or by permeation through intercellular spaces it is taken up in the submucosal lymphatic plexus and carried to regional lymph nodes for disposal Depending on the virulence of the invader this may or may not be successful

The significance of the normal mucosa in protection against pathogenic bacteria may be illustrated by infections caused by pneumococci These potentially virulent cocci are commonly carried in the normal pharynx but do not invade and cause disease unless the normal mucosal barrier has been damaged by a viral infection or irritant gas The nature of the damage is not known except that necrosis of ciliated epithelial cells occurs accompanied by an acute inflammatory reaction

The influence of alterations in the mucosa which do not proceed to cell destruction or gross inflammatory changes may have a significant bearing on the incidence of streptococcal disease in geographic areas such as the eastern slope of the Rocky Mountains Rammelkamp and his associates (reviewed by Rammelkamp 1955 56) have shown that the dried but viable organisms prevalent in the environment have little capacity to cause infection and that the reasons for high endemicity should be sought elsewhere It is not illogical to consider that mucosal alterations incident to living in a climate characterized by very low relative humidity and low environmental temperatures during the winter months with chronic irritation of the respiratory mucosa may reduce its ability to prevent implantation of streptococci

From the above discussion it is apparent that our knowledge of the normal defenses of the nasopharyngeal mucosa is rudimentary and that careful analysis may contribute much to the prevention of respiratory infection

When pathogenic bacteria produce infection *of the lower respiratory tract they are usually* found also in the upper respiratory passages For this reason posterior nasal or nasopharyngeal cultures give better information than those taken from the intermediate oropharynx which is lined with squamous epithelium Protection of the *trachea* and the *bronchi* appears to depend on mechanisms similar to those in the nasopharynx The cilia of the epithelial cells are important in removal of particulate matter although in the presence of a viscous mucus containing inflammatory exudate their effectiveness may be so reduced that pathogenic bacteria are able to reach the lungs by direct extension Lymphatic drainage from this area appears to be less than that of the upper respiratory tract although in certain infections such as inhalation anthrax of experimental animals early and massive involvement of the paratracheal and peribronchial nodes occurs Knowledge of the antibody content of tracheobronchial exudates is fragmentary However it has been found that sputum may contain antibodies to influenza virus

Pathogenic bacteria may reach the lungs and cause infection either by direct extension from the upper respiratory tract or through the inspired air By the use of simulants as well as by direct study of bacterial infections in experimental animals it has been shown that penetration to the alveoli depend upon particle size Particles of 5 microns or smaller can readily reach the alveoli through inspired air whereas larger particles are filtered out The implications of these observations in the study of pulmonary disease are great especially as they relate to infections such as pulmonary tuberculosis Q fever and possibly also to laboratory or industrial infections caused by *Brucella* The alveoli normally contain mononuclear phagocytic cells and are copiously supplied with lymphatics which remove foreign material to regional nodes Furthermore they are completely surrounded by blood capillaries from which phagocytes antibody and other humoral factors can quickly enter them In contrast with the nasopharyngeal area the number of organisms

present in the lower respiratory tract is very small indeed in normal man the lungs are sterile despite the fact that inspired air uniformly contains micro organisms in large numbers and before reaching the lungs has passed across mucous membranes that are heavily seeded with many microbial species The efficiency of the defense mechanisms of the *normal* upper respiratory tract is emphasized by the fact that acute pulmonary infections caused by virulent pathogens such as pneumococcus *Streptococcus pyogenes* and staphylococcus almost invariably are secondary to damage to the upper respiratory tract caused by viral infections such as influenza common colds and measles or follow inhalation of a noxious gas or anesthetic

The different regions of the *alimentary tract* from mouth to anus have a diversity of microbial inhabitants more or less characteristic for each region and the infections that occur reflect the effect of local conditions The *mouth* is lined with a squamous mucous membrane and possesses an extensive system of lymphatic drainage The secretions from the mucous membranes and the salivary glands have antimicrobial properties dependent upon pH the presence of lysozyme and other humoral factors including components exuded from the blood The mechanical flushing action of the secretions functions to prevent local accumulations of micro organisms and their colonization which however may be interfered with by partially occluded areas such as are caused by abnormal dentition The studies of Bloomfield (1922) show that particles entering the mouth are efficiently swept toward the base of the tongue and swallowed

The flora of the *oropharynx* differs from that of the mouth and is more akin to that of the respiratory tract Under normal conditions the tonsils which are lymphoid organs presumably play a large part in disposal of infectious agents in common with other components of the lymphatic system Realization of their protective role has diminished greatly the indiscriminate practice of tonsillectomy in children Occasionally however the tonsillar crypts may become the site of chronic infection by dangerous pathogens such as Group A streptococci and diphtheria bacilli which are extremely difficult to eradicate by treatment with antimicrobials Under such conditions tonsillectomy has been recommended both for the patient s own welfare and to prevent spread of the bacteria to other persons Recently it has been shown especially through the studies of Huebner and his associates that the lymphoid tissue of the nasopharynx and the oropharynx commonly harbors in a more or less latent condition viruses belonging to the large group known as adenoviruses The significance of the chronic carriage of these human pathogens in the epidemiology of the diseases caused by them has not been defined

Most of the micro organisms that are swallowed are killed in the *stomach* because of the acidity of gastric juice However the acid environment is only a partial barrier, since the agents of bacterial diseases such as salmonellosis shigellosis and cholera and viral diseases such as poliomyelitis and infectious hepatitis are acquired by ingestion Passage through the stomach in a viable state would appear to be facilitated by neutralization of acid by food Other bacteria such as tubercle bacillus have little difficulty in surviving in the stomach even under fasting conditions so that gastric washings are commonly used to demonstrate tubercle bacillus originating from bronchopulmonary lesions in patients from whom sputum sp cimens cannot be obtained If gastric acidity is reduced as in pernicious anemia or cancer of the stomach yeasts fungi and other micro organisms proliferate actively

The *small* and the *large intestines* contain an extensive and varied flora whose nature depends upon a variety of factors only a few of which are understood Certainly microbial antagonism is one of the e so that if alterations in diet are made the outgrowth of one species may be favored at the expense of others similarly if large do es of broad spectrum antibiotics are administered susceptible species may be virtually eliminated from the gut with resulting overgrowth of resistant bacterial species In some cases it has been maintained that suppression of the normal intestinal flora by prolonged use of antibiotics has led to infection caused by resistant organisms that are normally held in check by bacterial antagonisms However evidence supporting this claim is not particularly convincing

An analysis of the part played by compo

nents of the intestinal wall and adjoining structures in preventing invasion by intestinal bacteria has been made by Miller and his associates from the study of mice exposed to ionizing radiations (Gordon et al 1955) Following exposure to 700 r total body irradiation injury to the mucosa is most severe on the day following irradiation but invasion of intestinal bacteria does not occur at this time presumably because one of the chief barriers to invasion the submucosal lymphoid tissue is still relatively intact By the third day the submucosal lymphoid tissue and the mesenteric lymph nodes have atrophied markedly the number of circulating leukocytes is reduced sharply and the intestinal bacteria can be recovered from regional nodes and from spleen and liver The damage to these defenses has permitted the bacteria to gain access to the blood stream despite the fact that the integrity of the mucosal lining has been practically restored by this time The reticuloendothelial system of spleen and liver is able for a further period of 1 or 2 days to maintain the sterility of the general circulation but eventually may be overwhelmed also From the observations of Miller and his associates it appears that the integrity of the mucosal coat is of less importance in preventing invasion than local and regional lymphoid tissue and circulating phagocytes

The participation of humoral factors in protection against invasion by bacteria from the gut lumen is not understood although it should be noted that the level of properdin which is active against gram negative enteric bacteria is sharply depressed by ionizing radiations (Ross 1956) Antibody to various enteric pathogens has been extracted from feces However it has not been proved that this so called coproantibody is of significance in protection against surface infections of the intestinal tract such as bacillary dysentery nor can this be determined until the nature of immunity to dysentery infections has been discovered

The studies of Ørskov and his associates in 1928 on the pathogenesis of fatal bacteremia in normal mice following feeding of a virulent strain of *Salmonella* bear a striking resemblance to those of Miller et al who employed avirulent bacteria in irradiated mice Ørskov showed that the virulent bacteria appeared in mesenteric lymph nodes soon after inoculation A short time later they were found in liver and spleen and subsequently are released into the general circulation It is apparent therefore that the intestinal mucosa does not provide a very effective barrier to invasion and that the regional lymphoid tissue constitutes a first line of defense

THE ROLE OF THE LYMPHATIC SYSTEM

Upon penetration of the external limiting membranes by infectious agents a variety of cellular and humoral defense processes come into play Beneath the epithelial surfaces there lies an extensive network of anastomosing capillary and lymphatic channels which are lined by endothelial cells The blood capillaries are relatively impermeable to foreign particles introduced into the tissue spaces but the lymphatics are permeated readily by large molecular substances by inert particles by bacteria and even by particles as large as red blood cells These foreign materials are carried quickly by afferent lymphatics to the lymph nodes draining the area of introduction propelled by active movement of the body by pulsation of the blood vessels or by the increased local pressure caused by inflammation

The lymph nodes exert a highly efficient though incomplete filtering activity which serves to localize and contain the infectious agent Filtration depends upon the complex branching of channels within the node and upon the phagocytic cells which line the sinusoids If the pathogen is highly virulent its retention in regional lymph nodes may be relatively brief before it appears in the efferent lymph As shown by the studies of Wood and his associates on pneumococcal infections in rats the efficiency of filtration and local disposal may be enhanced considerably if an inflammatory reaction is already present in the node (reviewed by Wood 1951 52) The presence of greatly increased numbers of active polymorphonuclear phagocytes in the inflamed node permits much more efficient removal of the bacteria

Up to the present time it is not clear how the *inflammatory reaction* at the site of introduction of infectious agents serves to localize the infection There are claims on the one hand that lymphatic blockade occurs because of formation of fibrin clots within the lymphatics and that this process inhibits carriage to regional nodes On the other hand there is good evidence that the inflammatory process

causes an increase rather than a decrease in lymph flow (see Rich 1936) The localizing effect of the acute inflammatory reaction in which as the studies of Wood indicate the regional lymph nodes participate more actively than if not inflamed, depends upon multiple factors the increased circulation to the area brings an increased number of circulating phagocytes many of which pass through the walls of the capillaries and are held locally by chemotactic influences that as yet are poorly understood deposition of fibrin inhibits extension through tissue spaces and enhances phagocytosis by providing a surface that enables the phagocytes to operate more effectively (Wood 1951 52) humoral factors such as antibody properdin and complement can achieve an increased local concentration due to increased glycolytic metabolism of host cells there occurs an increase in organic acids especially lactic acid which prevent growth and may even kill many types of micro organisms (for review see Dubos 1954) upon death and dissolution of phagocytes various antibacterial substances such as lysozyme basic polypeptides and long chain fatty acids are released In addition the increased local temperature may affect adversely the multiplication of the parasite while accelerating the defense functions

Whether inflammatory reaction does or does not exert a protective effect has been a subject of discussion for many years Much of the discussion has centered around the influence of the delayed allergic inflammatory reaction upon immunity to tuberculosis Although the evidence available indicates that allergic inflammation does not prevent *implantation* of tubercle bacilli it does appear to assist in localizing the infection once established Treatment of tuberculous patients with cortisone depresses the local inflammatory reaction and rapid extension may occur unless controlled by chemotherapy While it is not absolutely proved that the detrimental effect of cortisone is caused by depression of inflammation this would seem to be a possible explanation A similar enhancing or evoking effect of cortisone on many infectious processes of man and animals caused by bacteria fungi protozoa and viruses have been observed (reviewed by Thomas 1952)

An additional and most significant function of the lymphatic system is that of antibody synthesis within the nodes and the spleen There is abundant evidence that if an antigen is introduced peripherally, antibody appears first in efferent lymphatics of the node draining the site of antigen introduction and only later appears in the circulating blood The concentration of antibody in efferent lymph is always higher than that found in the general circulation Moreover it has been shown that the cells of lymph nodes of animals which have been injected with an antigen produce antibody upon extirpation and injection into animals of the same species Therefore the evidence is clear that lymphatic structures are important sites of antibody production In acute bacterial infections antibodies usually do not appear in the general circulation for several days after the disease has begun On the other hand it should be borne in mind that the antibody found in the blood represents the excess that has been spilled forth from the lymphoid organs which as we have seen form the primary barrier to extension from the site of implantation of the infectious agent Obviously formation of antibody in the lymph nodes has taken place before it can be detected generally indeed in diseases such as pneumococcal pneumonia where the relation of antibody production to recovery has been extensively studied termination of the acute disease occurs about the time when antibody is first demonstrable in the circulation Before that time the regional lymph nodes have been well supplied with antibody produced within them and the crucial struggle between micro organism and host may well have taken place there and not in the presenting lesion

Removal of Micro organisms From the Blood Stream

Lymph borne bacteria may traverse one or several interposed lymph nodes before they reach the venous circulation by way of the thoracic duct If they are to produce a generalized blood borne infection they must be present in the circulation in sufficient numbers to overcome the cellular and humoral defenses of the blood as well as the highly effective splanchnic clearing system localized in the reticuloendothelial cells of liver and spleen

In the dog which is a naturally resistant species Gregg and Robertson (1953) have shown that the pneumococcidal activity of the blood itself which is dependent upon natural opsonins and phagocytosis is the principal mechanism limiting bacteremia in experimen

tal infection On the other hand in the rabbit a naturally susceptible species the phagocytic cells of the reticuloendothelial system are chiefly responsible for clearing the blood In experimental staphylococcal infections in both dogs and rabbits it has been shown that the bacteria are removed principally in the reticuloendothelial system and little or not at all in the peripheral circulation

Martin and Kerby (1950) found that staphylococci are removed from the splanchnic circulation much more efficiently than pneumococcus Even in animals dying of pneumococcal infection the efficiency of splanchnic removal of both pneumococci and staphylococci was not diminished so that exhaustion of splanchnic clearance could not be demonstrated It should be noted that of the microorganisms tested virulent pneumococci were cleared at the slowest rate so that the balance between bacterial multiplication and the splanchnic clearing mechanism is in favor of the former If specific antibody is present splanchnic removal of pneumococci is much enhanced

The virulence of the infecting bacteria plays an important part in the efficiency of splanchnic clearance In Wright's studies (1927) moderately virulent and highly virulent pneumococci injected in rabbits were cleared rapidly during the first 5 hours A transient increase in bacteremia occurred between 5 and 24 hours in the case of the strain of intermediate virulence but thereafter it was controlled However multiplication of the highly virulent strain in the host tissues is so rapid that the clearing mechanism is unable to cope with the large number of organisms so that overwhelming bacteremia occurs and death ensues The fatal outcome is determined not by a failure of the clearing mechanism which continues to function at seemingly unreduced efficiency but rather to multiplication of virulent organisms at a rate which the clearing mechanism cannot meet

In addition to the splanchnic clearing mechanism which is carried on by Kupffer cells in the liver and the macrophages lining the sinusoids of the spleen significant numbers of bacteria may be trapped and ingested by polymorphonuclear leukocytes in alveolar capillaries of the lungs Most of the laden granulocytes appear then to be transported to the reticuloendothelial cells for final disposition

Surface Phagocytosis Wood (1951 52) has described ingestion of bacteria in peripheral capillaries by the process he has termed surface phagocytosis Phagocytes in the inflamed area become sticky and adhere to the capillary endothelium over which they move freely and have increased capacity to ingest bacteria presumably because of a greater mechanical advantage The studies of Wood have directed renewed attention to the importance in the early defense against infection of phagocytosis *that takes place in the absence of specific* opsonizing antibody As Wood has demonstrated the nature of the surface is of crucial importance on a smooth surface such as the visceral peritoneum or the normal capillary endothelium phagocytosis in absence of antibody is almost nonexistent However if the surface is roughened as by fibrin deposits collections of phagocytes in sinusoids of lymph nodes or by granulocytes adhering to capillary walls in inflamed areas the polymorphonuclear leukocytes are able to pin down and engulf encapsulated bacteria that resist phagocytosis on smooth surfaces The pulmonary alveoli similarly appear to offer a surface appropriate for phagocytosis in the absence of opsonins

When specific antibody makes its appearance the nature of the surface makes little or no difference The antibody coated bacteria become sticky adhere to the phagocytes and are ingested readily

Multiplication of Bacteria Within Phagocytes Up to the present point the discussion of the clearing mechanism has assumed that ingested bacteria are destroyed by the phagocytes whether of fixed or free wandering type While this is true for many bacteria that cause acute infections there are numerous cases in which the ingested microbes both bacteria and fungi multiply within the phagocyte causing its eventual destruction and release of increased numbers of micro organisms In infection by tubercle bacillus or by *Brucella* the chief site of multiplication is within mononuclear phagocytes Similarly in infection by *Salmonella typhosa* growth of the bacteria takes place chiefly inside the cells Avirulent staphylococci are killed within phagocytes but as Rogers and Tompsett (1952) have shown virulent coagulase positive staphylococci that have been phagocyted grow in microcolonies inside the leukocytes which are then destroyed Therefore growth within

phagocytic cells plays an important part in the pathogenesis of certain infections In the *secluded haven of the parasitized phagocyte* the bacteria are able to grow in isolation from the humoral defenses The factors that participate in the ultimate destruction of these intracellular parasites are unknown

NONSPECIFIC ENHANCEMENT OF PHAGOCYTIC ACTIVITY

As shown originally by Lurie in 1942 (for review see Lurie 1950), mononuclear phagocytes derived from a tuberculous animal or an animal immunized with BCG exhibit increased phagocytic activity not only for tubercle bacilli but likewise for unrelated particulate material such as carbon and collodion particles Two other related effects have also been observed In the tuberculous animal monocytes are mobilized more quickly upon injection of tubercle bacilli or mineral oil as compared with the normal Moreover the monocytes from tuberculous animals are more resistant to the toxic effect of the ingested bacilli and there is greater destruction or inhibition of growth of tubercle bacilli in the resistant mononuclear cells

The effects enumerated above are independent of conventional antibodies and appear to result from generally increased physiologic activity of the mononuclear cells in tuberculous animals Aside from the interference immunity possessed by certain virus infected cells to infection by other related or unrelated viruses the observations on the increased resistance of mononuclear phagocytes to tubercle bacillus independent of serum antibody, are the most convincing evidence so far obtained of the existence of *cellular immunity*

Recently Elberg Schneider and Fong (1957) have shown that monocytes from animals vaccinated with BCG are more resistant to tubercle bacillus and also to *Brucella melitensis* The converse was observed also in animals vaccinated with *Brucella* Elberg and associates are inclined to attribute the cross immunity to the fact that both pathogens have an intracellular habitat However such a correlation does not explain the increased phagocytic activity of monocytes from tuberculous animals toward inert particles such as carbon or collodion

NONSPECIFIC CHANGES IN HOST RESISTANCE

In the preceding section observations on nonspecific increases in cellular immunity have been summarized Although most of these studies have been carried out on isolated cell systems similar effects are operative in the intact animal Moreover, in addition to nonspecific increase in resistance, equally unspecific *decrease* may also occur How much of both the decrease and the increase may be due to cellular or humoral factors is not yet clear, although without doubt both are operative

That the susceptibility of animals to infection can be diminished nonspecifically by *injecting killed bacterial cells or constituents* thereof was described about the turn of the century by Almroth Wright in his studies of typhoid immunization Wright observed a negative phase of resistance that preceded the development of immunity following vaccination In recent years considerable light has been thrown on these early observations particularly through study of the nonspecific effects of bacterial endotoxic O antigens on resistance and their relationship to the properdin levels of blood

Employing *E coli* as test organism Rowley (1956) has shown in mice that soon after injection of the lipopolysaccharide fraction of the O antigen there is a sharp decrease in the bactericidal action of the serum which is not maintained but to the contrary is followed within 24 hours by a marked increase in bactericidal power which persists for a week or more The resistance of the animals to experimental infection is increased at the same time Injection of zymosan a poorly soluble carbohydrate fraction obtained from yeast and of high molecular weight dextrans produces similar effects Landy and Pillemer (1956) have shown that injection of lipopolysaccharides from a variety of gram negative bacterial species increases resistance to a number of gram negative pathogens It has been found that the early decrease in bactericidal power of the blood serum is associated with a fall in properdin levels and that later when the bactericidal power of the serum and resistance to infection increase there is a concomitant rise in properdin levels which persists throughout the period of increased resistance

While it is tempting to attribute these fluc

tuations in resistance solely to the action of the properdin complement system it is apparent particularly from studies of Dubos and Schaedler (1956) that this is an oversimplification The properdin complement system has been shown to be bactericidal in vitro only for gram negative bacteria However Dubos and Schaedler have reported that following injection of purified endotoxic lipopolysaccharide in mice there is a considerable enhancement of resistance to infection by staphylococci and tubercle bacilli that persists for periods of time which vary directly with the amount of lipopolysaccharide injected Parenthetically it should be noted that Landy and Pillemer did not observe increased resistance to the same strain of staphylococcus following lipopolysaccharide injection There were at least two differences in the experimental procedures used by the two groups of investigators One was that they worked with different strains of inbred mice The other was that Landy and Pillemer used the intraperitoneal route of infection whereas Dubos and Schaedler used the intravenous route It seems probable that other important changes in addition to alterations in properdin levels play a role in the accentuated resistance From the original studies of Lurie and those of Elberg and his associates on the increased phagocytic capacity of mononuclear cells for tubercle bacillus following immunization with endotoxin containing *Brucella* it seems not unlikely that the increased resistance to infection by staphylococcus and tubercle bacillus observed by Dubos and Schaedler may depend in part at least on a nonspecific increase in the activity of phagocytic cells

In addition to the effects of endotoxic lipopolysaccharides nonspecific changes in resistance to infection can be brought about by steroid hormones and by exposure to radiation as discussed previously Depression of resistance may also follow administration of salts of organic acids and inhibitors of certain metabolic steps of the Krebs cycle (for review see Dubos 1954) Dubos and his associates have made extensive studies on the effect of dietary alterations upon resistance of mice to bacterial infections (reviewed in Dubos 1954 Schaedler and Dubos 1956 1958) Employing staphylococcus K *pneumoniae* and tubercle bacillus as test organisms they have reported that diets qualitatively or quantitatively deficient in fats amino acids or vitamins administered for 5 to 7 days prior to infection cause a decrease in resistance The effect was found to be reversed by feeding an adequate diet for a period as short as 1 to 3 days A finding of great interest is that of Schneider and Zinder (1956) who have reported that a methanol soluble factor present in certain natural foodstuffs causes an increase in survival of mice infected with Salmonella

Until recent years most of the investigations on resistance or immunity to bacterial infections have been concerned with the part played by specific antibodies and little attention has been devoted to nonspecific factors such as those described above A more rounded picture of the host s resistance on first contact with an infectious agent would perhaps allocate specific immunity to an emergency role that comes into play only after the natural mechanisms of resistance whether genetically determined or modified by environmental changes have failed to eradicate the invader

Humoral Factors in Resistance to Infection

Specific Antibodies Up to the present time the evidence indicates that specific antibodies are formed in plasma cells and perhaps also in other cells of the mesenchymatous lymphocyte macrophage system chiefly though not exclusively in lymph nodes and in the spleen From a teleologic viewpoint synthesis of antibody in lymphatic structures is highly appropriate since as has been described above the lymphatic system constitutes the initial barrier to invasion of the internal organs once infectious agents have penetrated the external or the internal epithelial membranes Our ideas of the role of antibody production as an early defense reaction have been distorted because most of the measurements of antibody during or following acute infections have of necessity been carried out on blood serum Measurement of antibody in serum does not give a true picture of antibody synthesis and concentration in lymphoid tissues because the amount present in the circulation represents the excess which has spilled over from sites of synthesis It follows that an effective concentration of antibody is pre ent in the lymph nodes considerably earlier than it can be demonstrated

in the blood in which moreover, it is much diluted and therefore more difficult to measure

Normal rabbits injected intravenously with heat killed pneumococci may show anticapsular antibody in their serum as early as 72 to 96 hours following intravenous injection of the antigen and active immunity to infection by virulent homologous organisms is demonstrable as early as 48 hours following antigen introduction (Curnen and MacLeod 1942) These observations serve to indicate that antibody production following primary antigenic stimulation can occur very promptly and that it should not be thought of only as a late manifestation of resistance to infection

If an animal has had previous exposure to an antigen the response is dramatic on reexposure The synthesis of antibody and its release into the circulation begin almost immediately the concentration in the blood rises steeply through the first few days and reaches levels often far above those seen on primary immunization This accelerated reaction variously called the secondary booster or specific anamnestic response in all probability is an important mechanism for defense against second attacks of disease The capacity to give a secondary immune response is an enduring attribute and may persist throughout life Its mechanism is not understood but some form of memory presumably cytoplasmic is possessed by cells that produce antibody Various theories have been advanced to account for this phenomenon but experimental evidence to support them is lacking (reviewed by Burnet and Fenner 1949) (see Chap 5)

Antibodies are γ globulins which combine with the specific antigen that caused their production or with related antigens Bacteria contain a variety of distinct antigens and following infection or injection of the whole micro organisms antibodies to many of the constituents are found in the blood serum During or following infection by Group A streptococci antibodies may be found to the M protein the C carbohydrate streptolysin O hyaluronidase desoxyribonucleases streptokinase erythrogenic toxin and many other components of the cell While the character of the disease may be influenced by antibodies to some of these cell components or products immunity to infection depends upon antibodies to M protein Similarly in the enterobacteria the relatively weak immunity to infection appears to be due to antibodies to the O antigen and in some cases, such as the typhoid bacillus also to a second substance called Vi antigen Antibodies to many other cell components including the flagella are formed as a result of infection or artificial immunization Demonstration of the appearance of antiflagellar antibodies or their increase in titer during a febrile illness indicates that the patient may be suffering from typhoid infection although these antibodies bear no relationship to specific immunity to infection Similarly aid in diagnosing streptococcal disease may be got from measuring antibodies to streptolysin O although these antibodies have no demonstrable role in protection against the disease

From consideration of these two examples it is clear that the presence of antibodies to a cell constituent may have little meaning with respect to immunity to infection Furthermore even though antibodies to the significant pathogenic components of a micro organism are present this does not guarantee immunity to the possessor since their titer may be insufficient Protection against infection by influenza viruses in man appears to involve not simply the presence of specific antibody in the blood but also requires a certain titer The explanation offered is that influenza is a surface infection and in order to protect the epithelial cells the surface of the mucosa needs to be bathed in antibody to prevent virus attachment Francis studies have shown that the amount of influenzal antibody in nasal mucus is only about one tenth to one twentieth that of blood so that if the levels in the blood are low the quantity that finds its way to the surface of the respiratory mucosa may be so small that it does not afford protection

Most humans have antibodies in their blood to pneumococcus type 7 capsular polysaccharide These so called 'natural' antibodies which are present generally in low titer agglutinate type 7 pneumococci in vitro and protect mice against experimental infection (MacLeod and Roe 1947) Paradoxically they do not uniformly protect man against pneumonia caused by pneumococcus type 7 since patients who are acutely ill with this disease commonly possess type 7 antibody when first seen It must be admitted that the situation with type 7 'natural' antibody in man is somewhat unusual and not readily explained but it is cited to emphasize that presence of anti

body, even to a significant pathogenic factor of the microbe and immunity to infection do not always coincide.

The origin and the functions of *natural antibodies* have been much discussed, but relatively little work on their characterization and significance has been carried out in recent times except for the studies on properdin which undoubtedly is an important component of the natural bactericidal activity of serum for certain gram-negative bacteria as well as having antiviral activity. The lack of specificity of properdin, certain of its physical properties and its biologic behavior in a number of respects suffice to remove it from the category of antibodies as the term is commonly understood.

Whether or not the natural antibodies provide innate protection against infection is far from clear. Two principal theories have been advanced to explain their production. The first is that synthesis of natural antibody globulins is determined genetically and does not require the presence of the antigen. These globulins present in most members of a species after a certain period of growth or maturation, because of their configuration react with particular antigens. The alternative explanation and one which is more susceptible to experimental demonstration, is that natural antibodies, including the iso-agglutinins, arise because of undetected exposure to the specific antigen itself or to an antigen closely enough related so that they cross-react. The latter is a very common phenomenon among polysaccharide-containing antigens derived from natural sources and may explain the origin of many of the natural antibodies. Thus although humans carry pneumococcus type 7 very infrequently (less than 1%), between 80 and 90 per cent of the population between the age of 1 year and 90 years possess antibodies. Viridans streptococci commonly carried in the throat have cross-reacting surface antigens and their presence can easily account for the occurrence of "natural" type 7 antibodies. Antibodies to pneumococcus type 2 are frequent in the serum of man and animals. Once again polysaccharides that cross-react with the type 2 polysaccharide are very common in nature, so that it is not surprising to find natural antibodies reacting with this particular configuration or a related one. Cross-reacting antigens are widely distributed among pathogenic and nonpathogenic members of the enterobacteria, and because of the universal carriage in the gut of a number of coliform species containing antigens that cross-react with antigens of Salmonellae or Shigella, it might be expected that natural antibodies to the latter would be found frequently. Examples of this sort could be multiplied extensively.

It has been held that lability to heat at 56° C. for 30 minutes is characteristic of natural antibodies and that conventional antibodies which arise as a result of immunization are stable to heating at this temperature. However, application of this criterion to distinguish between natural and immune antibodies does not appear to be justifiable. It should be recalled that the precipitating power of human and certain animal diphtheria antitoxins is destroyed by heating whole serum at 56° C. (Cohn and Pappenheimer, 1949), but the capacity to neutralize toxin is impaired only slightly. Heated human antitoxin can still combine with complement in the presence of toxoid and retains its ability to sensitize guinea pigs passively to anaphylactic shock. It has been shown that the effect on precipitation is caused by interaction between the antibody globulin and some factor present in serum albumin. Purified antitoxic γ-globulin showed no loss of precipitating ability upon heating at 56° C. Because of the known lability of properdin and complement in whole serum to heat at 56° C. and the destruction of the precipitating power of diphtheria antitoxic serum at this temperature, the validity of applying this test to distinguish between innate or natural antibodies and antibodies arising as a result of contact with a specific antigen remains doubtful.

Properdin. The presence of a previously unrecognized protein, properdin, present in the serum of man and animals was described by Pillemer and his colleagues in 1954, and its role in natural resistance to infection was indicated at that time. An extensive review is presented in the symposium entitled "Natural Resistance to Infections" (1956). Properdin occurs in the globulin fraction of serum of man and animals, the levels in a particular species being relatively fixed and characteristic of that species. It differs in many respects from conventional antibodies and requires the participation of all 4 components of complement and Mg^{++} in order to exert its hemo-

lytic bactericidal and antiviral activities Properdin is active against gram negative bacteria and appears to have little or no action against gram positives Following lethal or sublethal whole body irradiation the levels fall sharply Upon the injection into animals of zymosan, a carbohydrate from yeast and various other polysaccharides from diverse sources the titers are also reduced sharply and this is associated with enhanced suscepti bility particularly to infections by gram negative bacteria The lowered levels which occur immediately following polysaccharide injection are succeeded, usually within about 24 hours by a return of properdin to levels well above the normal Increased titers persist for a week or more, depending upon the amount of polysaccharide injected originally and during this period animals show increased resistance to certain infections

Numerous other antimicrobial factors that may be concerned in resistance to infection have been described in normal tissues and fluids of the animal body A description of them and their activities is beyond the scope of this chapter (for review see Skarnes and Watson 1957)

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5

Serology and Immunochemistry

The host parasite relationship outlined in previous chapters can be summarized by Theobald Smith s statement that it is An armed truce accompanied by predatory proc esses on both sides as opportunities present themselves The nature of the uneasy balance that constitutes infection the factors that tip it toward effective resistance or toward acute or chronic disease and the kinds of predatory processes all form the subject matter of the science of immunology In its applied aspects immunology is also concerned with the ways in which knowledge of these phenomena can be turned to the advantage of man his domestic animals or his farm crops by the prevention or the amelioration of infectious diseases

Although immunology is concerned with the total picture of infection and resistance its development like that of any science has been uneven and the depths of its sublines are de pendent on the relative availabilities of suit able technics of search Thus most of our data on the parasites which initiate an infection and the host responses to this invasion are concerned (1) with an over all biologic de scription of the events and (2) with an at tempt to implicate in these processes microbial antigens host antibodies complement or tis sue cells Ultimately however it should be possible to specify in detail the role of such factors as surface antigens for microbial viru lence of hyaluronic acid for tissue immunity or of phagocytes for cellular immunity in terms of the definite enzymatic or other chemical reactivities of well characterized molecules Much also remains to be learned concerning disturbances in host metabolism occasioned by infection, and the influence of microbial toxins on the so called bio chemical lesion of the host Although there is much scattered evidence that resistance is profoundly influenced by variables such as the age nutrition or heredity of the host (and often of the parasite) it has been difficult so far to relate these to specific physiologic mechanisms and the general problem offers fertile ground for future research

While immunology is obviously an eclectic cience it has not been without some develop ments of its own Thus the serologic technics to be discussed below have been applied not only to the specific problems of infectious dis ease but also are finding increasing applica tion to other problems of pathology (blood group incompatibilities cancer collagen dis eases) to problems of general biology (evolu tion of micro-organisms tissue transplantation reactions the sequence of embryologic devel opment) and to biochemistry itself (struc tures of proteins from various species en zymatic adaptation etc)

The bearing of serology on the problems of immunology should already be evident How ever in becoming proficient in the use of sero logic tools it is necessary that the student understand fully the limitations as well as the advantages of the antigen antibody approach in the total pattern of infection and resist ance and distinguish in each instance the

serologic reactions that are useful only in characterizing a micro organism or in signifying its pre ence during past or present infection from those that have a real influence on host resistance

Varied aspects of the literature of immunology including some of the historical aspects are presented in the books of Wilson and Miles (1955) Cushing and Campbell (1957) Boyd (1956) Raffel (1953) and Perla and Marmorston (1941) Landsteiner has summarized his extensive contributions to serology in a monograph (Landsteiner 1945) and the field of experimental immunochemistry is well covered in the books of Kabat and Mayer (1948) Cohn (1952) and Heidelberger (1956) References to other reviews and notable papers including developments in allied fields are provided by chapters in the Annual *Reviews of Microbiology of Biochemistry of* Physiology and of Medicine or the series of *Advances in Protein Chemistry in Carbohydrate* Chemistry and in Genetics Valuable symposia are also held at intervals by scientific societies

VARIABILITY

The phenomena of infection and resistance cannot be well understood without an appreciation of the fact that however superficially alike living things may seem to be if they are examined closely individual differences appear Thus if a group of animals (or humans) is challenged in an experimental or natural infection some individuals will die following relatively low dosages while others will survive much greater amounts The group behavior may be summarized by the mean and by the spread (standard deviation) A portion of the group response may be constitutional and thus relatively permanent since the mean response may often be shifted upward by selective breeding for resistance However a significant portion is transient and the resultant of temporary chance combinations of many factors favorable or unfavorable to the individual As a consequence if the survivors from the first challenge are collected and graded challenges again administered some individuals will now be found to be susceptible and will succumb to lower dosages than they withstood previously This is usually difficult to demonstrate for infectious agents since by virtue of the immunization exposure to the fir t challenge individuals may gain antibodies which introduce a new complication However it can be demonstrated for both animal and microbial populations exposed to certain drugs to which tolerance is not readily acquired

If a population is actively immunized with an antigen under the most uniform conditions obtainable it can be shown by serum titrations that the antibody responses are not uniform for all individuals but form a distribution over the population Thus even if the antibody alone determined resistance a uniform antigen administration would not result in a uniform increase in resistance That antibody is not the sole factor in resistance can also be demonstrated for if instead of requiring that each animal manufacture its own antibody—a response we have seen to be a variable one—we may passively donate to it an aliquot of serum from a donor pool Every animal thus begins the test with an equal amount of antibody Nevertheless subsequent challenge reveals the same substantial variation (if not the same mean resistance) as follows active immunization Thus either factors other than antibody contribute significantly to resistance or else animals may differ in the extent to which they can utilize the antibody they already have

With the proper design of experiments it is not only possible to take biologic variability into account for practical problems of immunology but also to learn more about infectious agents and host resistance The various technical tools necessary for serious study will be found in the books and the papers of Finney (1952) Batson (1951) and Treffers (1956)

ANTIGEN ANTIBODY REACTIONS

Characteristics

In each species of animal or micro-organism there are a number of substances in particular proteins and polysaccharides that possess chemical groupings more or less specific in arrangement If these substances termed *antigens* are introduced into an animal of a foreign species the latter produces in its serum a substance which reacts specifically with the antigen with the object of neutralizing and removing it The new component is termed *the specific antibody* It is a protein that has many of the properties of the other serum proteins of the species producing it

Although we shall return below to a more detailed consideration of the properties of

TABLE 6 PRECIPITATION OF RABBIT ANTIBODY TO CRYSTALLINE EGG ALBUMIN BY VARIOUS AMOUNTS OF THE LATTER ANTIGEN

TUBE NUMBER	SERUM	EGG ALBUMIN ANTIGEN ADDED	APPEARANCE OF TUBES AFTER 2 HOURS AT 0° C
	ml	*mg N*	
1	1 0	0 020	Slight precipitate
2	1 0	0 040	Moderate precipitate
3	1 0	0 080	Heavy precipitate
4	1 0	0 120	Heavy precipitate
5	1 0	0 200	Slight precipitate

antigens and of antibodies (including evidence that what we have termed antibody is not a single molecular species but rather a group of such molecules, all of which react with the antigens) it is necessary first to introduce a *number of concepts which can be illustrated* best in terms of antigen antibody interactions of which there are numerous manifestations Their characteristics may be developed by following through a typical experiment Sup pose that a single homogeneous protein, such as crystalline egg albumin is selected as anti gen and injected into a rabbit by an appro priate route usually intravenously Although antibody may often be demonstrated after a single injection sera of high antibody content are produced only after multiple injections totaling perhaps several mg of antigen

After the last injection the animal is per mitted to rest for a week or 10 days to allow the antibody content to increase to a maximum and then is bled The serum is carefully sepa rated from the clot clarified by centrifuga tion if necessary and then suitable amounts such as 1 ml portions are distributed into a *number of test tubes Next various amounts* of the egg albumin antigen are added as in Table 6 The contents of the tubes are mixed and allowed to stand for a time This may be 1 hr at 37° C or from 1 to 7 days at 0° C

The combination of antigen with the anti body contained in the serum is evidenced by a visible reaction—the formation of a specific precipitate The latter contains both antigen and antibody Serum from a nonimmunized animal, or from an animal injected with an antigen other than egg albumin will not give a reaction with any amount of the egg albumin antigen a reflection of the specificity of the reaction

As we shall see later, for many purposes the reaction need not be carried further than this if only a qualitative indication or a rough quantitative indication of antibody is needed For a quantitative assay however the tubes are centrifuged until the precipitates are well packed and the supernates carefully poured off and set aside for later examination Since the reaction was carried out in whole serum it is necessary to wash the precipitates several times with saline to free them of the large amounts of nonantibody protein that adhere When this is done the amount of antibody in the precipitates may be determined in one of several ways For example the precipitates might be dried and weighed but this is not convenient and is seldom done More easily advantage may be taken of the fact that as antibody is a serum protein containing nitro gen (N) the determinations can be made by one of the usual methods of estimating that class of substances such as the micro Kjeldahl method or the sensitivity can be increased by employment of one of a variety of colorimetric methods which can assay precipitates contain ing as little as 1 μg of protein N (McDuffie *and Kabat 1956) The data may be reported* directly as mg (or μg) of protein N or as a color equivalent to a definite amount of a reference protein such as γ globulin The re sults for our test system expressed in terms of protein N are given in Table 7 if it is desired to express these as mg of protein the N values can be multiplied by the factor 6 25

We have mentioned that the specific pre cipitates consist of antigen and antibody with out detailing the proportions of each or men tioning whether the precipitates contain all of the antigen or antibody present in the system To interpret the data it is necessary to have information on the latter points and we shall need the supernates which were poured off

TABLE 7 TOTAL PROTEIN AND ANTIBODY PROTEIN PRECIPITATED AS DETERMINED BY THE MICRO KJELDAHL ANALYSES*

TUBE NUMBER	SERUM	EGG ALBUMIN ANTIGEN ADDED	TOTAL PROTEIN PRECIPITATED	ANTIBODY PROTEIN PRECIPITATED†	TEST ON SUPERNATE
	ml	mg N	mg N	mg N	
1	1 0	0 020	0 303	0 283	Excess antibody only
2	1 0	0 040	0 539	0 499	Excess antibody only
3	1 0	0 080	0 813	0 733	Neither antibody nor antigen
4	1 0	0 120 (only 0 087 precipitated)	0 730	0 643	Slight excess antigen
5	1 0	0 200 (only 0 048 precipitated)	0 414	0 366	Large excess antigen

* Modified by interpolation from Heidelberger M and Kendall F E 1935 A quantitative theory of the precipitin reaction III The reaction between crystalline egg albumin and its homologous antibody J Exper Med 6 697 720

† By difference between total protein N precipitated and antigen N precipitated

from the precipitates after the initial centrifugation

Each supernate is distributed between 2 test tubes To 1 tube we add a minute quantity of the antigen egg albumin A precipitate would indicate an excess of free antibody in the supernate To the other tube we will add a small amount of the immune serum A precipitate here indicates an excess of antigen in the original supernate As is evident in the last column of Table 7 the supernate of tube No 3 yielded negative tests for both antibody and antigen which is interpreted to mean that the amount of antigen originally chosen 0 08 mg egg albumin N was just sufficient to remove all of the antibody without leaving an excess of antigen in the supernate In practice it is found that if the amounts of antigen are spaced closely together more than one tube may show this behavior therefore such systems are spoken of as having an *equivalence zone* rather than an *equivalence point*

The data in Table 7 may be plotted as in Figure 16 Curve A represents the experimental findings—the amount of precipitate obtained for each amount of antigen used If the antigen had been a non nitrogenous substance such as certain bacterial polysaccharides curve A would also represent the amount of antibody precipitated for although the precipitate contains both antibody and antigen the latter would not register by the analytical technic used However in the example which we have used the precipitate contains antigen N as well and this must be deducted from the total N so that the antibody N will be given by the difference

It is a valuable characteristic of systems containing only a single antigen that in the region of excess antibody (such as tubes No 1 and No 2 of Table 7) and in the equivalence zone (which includes our tube No 3) all of the antigen added is precipitated by the antibody that comes down In the region of antigen excess (tubes No 4 and No 5) although the total N curve accurately expresses an experimental fact the antibody N curve cannot be obtained from it by subtracting the antigen N added since some of the latter remains in the supernate and its amount must first be assayed In the infrequent instances in which the antigen possesses a distinctive natural marker (such as copper or iron in the proteins hemocyanin or hemoglobin) the antigen may be estimated independently of antibody by direct determinations of the marker in either the precipitate or the supernate Through the modern use of radioactive tracer methods this principle can be extended to the tagging of almost any type of antigen or of antibody itself (Melcher Masouredis and Reed 1953) Finally in all cases the supernate containing the excess antigen can be treated as an unknown and set up with suitable amounts of the immune serum so that precipitates in the region of excess antibody

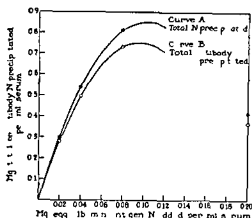


FIG 16 Total nitrogen and antibody ni trogen precipitated by Egg Albumin antigen from an immune rabbit serum The circles *are experimental the curves are calculated* from equations discussed on this page

will be formed By assay of their total N and reference to calibration charts such as Figure 16 the actual quantities of antigen in the system can be determined and the corrections necessary for the solution of our problem made

As is evident from the data for tubes No 4 and No 5, *the amounts of antibody precipi* tated decrease as larger and larger amounts of antigen are added beyond the equivalence amount, and with sufficient antigen excess there will be a complete inhibition of precipi tation In this region of partial or complete inhibition antigen and antibody combine to form soluble complexes and this property must be taken into account in titrations, else the zone of precipitation may be missed en tirely Some antigen antibody systems are not too sensitive to slight excesses of antigen which indeed may be necessary to remove the last traces of antibody *Under these conditions* the antibody curve (corresponding to B chart 1) exhibits a flat plateau before descending This is apparently a normal characteristic of antipolysaccharide antibodies it is also given by the diphtheria antitoxins in the sera of a number of immunized animal species However, Pappenheimer and his co workers have shown that in the latter instances it is due to multiple antibody antigen systems and that if the sera are first absorbed with preparations of the impurities normally present in most diphtheria toxoid samples, steeply descending curves result when the sera are then tested *against active diphtheria toxoid*

Since nearly all of the antigens employed in serology must of necessity be extracted from complex biologic systems difficult to fraction ate the serologist always welcomes informa tion on the possible heterogeneity of his test *material reaction curves that do not descend* readily with antigen excess serve as a con venient warning of a likely although not a *necessary heterogeneity Another indication* of multiple antigen antibody systems is pro vided if positive tests for ***both*** antigen and antibody are given by any one supernate tube *single systems given only the reactions tabu* lated in the last column of Table 7 (Cohn 1952)

The top of the antibody curve (B Fig 16) represents the maximum amount of antibody precipitable by the antigen under the condi tions chosen and is therefore a direct and *objective measure of the content of this par* ticular antibody in the serum

Heidelberger and his co workers have shown that the reaction curves for a wide variety of antigen antibody systems can be expressed by one or another of two equations and that the constants for these can be evaluated from any two accurately determined points up to the region of excess antigen Once these are avail able the amount of antibody that would be precipitated by any amount of antigen can be computed readily The *solid lines for Fig* ure 16 were drawn from a representative set of such computed values Although perfect agreement cannot be expected for all points the average deviation between the 9 calcu lated and experimental points listed in the original (Heidelberger and Kendall 1935) is *only 2 per cent with individual deviations* of from 0 to 8 per cent Further details on the use of these equations are given in Chapter 2 of the book by Kabat and Mayer (1948)

* (R) = rabbit (H) = horse (Hu) = human antisera Ea = chicken ovalbumin Pn = pneumococcus (with type designation) Other abbreviations elf-evident

† The antibody values are estimated from data taken under more or less representative test conditions With few exceptions the conditions were *selected for convenience rather than optimal sensitivity* and the latter presumably could be increased by varying these conditions

‡ Unless otherwise referred to in the text ob erver citations are not given in the bibliography

§ Sera noted as falling into one of the two indicated groups

TABLE 8 THE ANTIBODY REQUIREMENTS FOR ENDPOINTS IN SOME REPRESENTATIVE SEROLOGIC REACTIONS

Test	Species and Specificity of Antibody*	µg Antibody N for Test†	Observer‡
Fatal passive anaphylactic shock in guinea pigs only cross reactive antibody utilized	(R) Pn 3 8	100 350	Kabat Coffin and Smith 1947
Ibid utilizing homologous antibody	(R) Ea or Pn 3	30	Kabat and Landow 1942
Anaphylactic contraction of isolated uterine horn	(R) Ea or Pn 3	0 01	Kabat and Landow 1942
Passively transferred Arthus reaction in rabbit minimal	(R) Ea	25	Fischel and Kabat 1947
Mouse protection passive 50% mortality	(H) Pn 1	1 8§	Goodner and Horsfall 1937
		1 3	Goodner and Horsfall 1937
	(R) Pn 1	0 8	Goodner and Horsfall 1937
Bacterial agglutination	(R) *S aureus*	2 5	Umezawa 1948
	(R) Rough Pn	0 3	Heidelberger and Kabat 1934
	(R) *Str pyogenes*	0 1	Henriksen and Heidelberger 1941
	(R) *S typhosa* O	0 02	Gurevitch and Ephrati 1947
	(R) *S typhosa* O	0 06	Muschel and Treffers 1956
	(Hu) *S typhosa* O	0 02 0 10	Gurevitch and Ephrati 1947
Precipitin reaction			
Visual	Various	2 5	Estimated
Colorimetric assays	Various	1 5	McDuffie and Kabat 1956
Special micro assays	Various	0 03	McDuffie and Kabat 1956
Complement fixation			
Quant 50% hemolysis	(R) Pn3	0 12 0 4	Osler Mayer and Heidelberger 1948
Visual complete hemolysis	(R) Pn3	0 12	Heidelberger Weil and Treffers 1941
Mazzini flocculation test			
4+ reaction	(Hu) purified Wassermann ab	0 08 0 4	Davis Moore et al 1945
	Ibid whole serum	0 03 0 15	
	(Hu) with purified cardiolipin antigen	0 014	Osler and Knipp 1957
Isoagglutination	(Hu) bl gr A or B	0 1 0 2	Kabat and Bezer 1945
Hemolysis per ml erythrocyte susp	(R) Forssman ab	0 05	Rapp 1953 in Osler and Hill 1955
Immune adherence test	(R) Pn 13	0 015	Nelson and Woodworth 1957 personal communication
Intradermal toxin neutralization test in rabbit skin	(R) Diphtheria toxin	0 012	Koshland and Englberger 1957
Passive cutaneous anaphylaxis in guinea pigs minimal reaction	(R) Ea	0 003	Ovary and Briot 1951
Turbidimetric growth assay for bactericidal activity 50% kill	(R) *S typhosa* O	0 001	Muschel and Treffers 1956

(*See footnotes on facing page*)

It is also possible to follow the course of precipitation by reading the initial turbidities in a photoelectric colorimeter Although the readings are expressed in arbitrary instrumental units the resulting curves are of the general form we have illustrated and can be converted to an absolute (weight) scale by a simultaneous Kjeldahl analysis on one point

TITERS AND OTHER QUANTITATION MEASURES

A variety of observational endpoints are used in serologic reactions Some are of the all or none type Thus in anaphylactic or in infection tests we determine the percentage of animals that die with characteristic symptoms following the administration of various challenge doses Erythrocyte hemolysis in complement fixation reactions may be estimated by the eye as incipient or as virtually complete On the other hand many serologic measurements are scored over their entire range When this is done by eye the results are often expressed on a scale running from 0 to ++++ with as many intermediate values as the observer feels he can recognize Alternatively as we have seen measurements may be made with an analytical balance titration assembly photoelectric colorimeter or other instrument When numerical values are obtained in most instances the dosage response curve can be expressed in equation form and the necessary constants evaluated from a few measurements Then further data may be derived by interpolation or extrapolation saving the experimenter the time and the materials necessary for their physical determination over the whole range desired In all cases appropriate statistical treatment of the data will enable the investigator to judge whether the differences found are valid or could result from chance variations Examples of this are given in the references cited above (p 115)

As is illustrated in Table 8 each serologic reaction requires a characteristic amount of antibody for its endpoint the latter may be a quite arbitrary one set at the convenience of the observer and if the reaction conditions are varied the antibody levels necessary may depart by a factor of 5 fold to 10 fold from the values tabulated

If we consider a reaction such as the agglutination test with hemolytic streptococci we may find that the degree of agglutination arbitrarily taken as the endpoint is reached with 0 1 μg *of antibody N (0 63* μg antibody protein) Since 1 ml quantities are used in the test, a serum that has 0 1 μg *antibody N* per ml evidently contains one endpoint unit per ml If the serum contained 1 0 μg of antibody N per ml it could be diluted *1 10 and* still provide the 0 1 μg of antibody N per ml of dilution necessary to give the endpoint Therefore, the amount that a serum must be diluted in order that it will just meet the endpoint requirement for antibody (reaction neither too strong nor too weak) will be in some proportion to the antibody content of that serum Serologists designate this dilution as the *titer* of the serum for the particular reaction under consideration A titer of 1 100 for our antistreptococcal serum thus summarizes the fact that this serum can be diluted 100 fold and yet 1 ml of this dilution will still give agglutination to the standard degree Such a serum will contain 10 times more of the antibody responsible for streptococcal agglutination than is possessed by a serum of titer 1 10

Table 8 also illustrates a second principle not only do tests such as anaphylaxis agglutination and bactericidal activity vary over a 1 000 fold range or more in their antibody requirements for their endpoints but even within a single type of test such as the agglutination reaction there may be a *125* fold or greater, difference in the amounts of antibody needed to agglutinate diverse organisms such as the staphylococcus and *S typhosa* An antistaphylococcal serum containing 1 0 μg of antibody N per ml would give only negligible or incomplete agglutination of the staphylococcus since *2 5* μg of antibody N are required for standard complete agglutination on the other hand an antityphoid O serum containing 1 0 μg of antibody N could be diluted about 1 16 since only 0 06 μg of antibody N is needed for the agglutination of *S typhosa* Furthermore although suitable quantitative data are not at hand there is good indirect evidence that even within the *S typhosa* system itself there are appreciable differences in the amounts of antibody needed to reach the agglutination endpoints via the O H or Vi specificities or more generally that somatic O agglutination requires substantially more antibody than does flagellar H agglutination This is indicated too in the consistently higher titers obtained with H sera although there is no reason to believe that they contain more antibody protein than do the lower titered O sera

From the above we may conclude that relative titers are valid measures of the relative antibody contents of sera provided that the sera being compared are all of the same specificity and are measured by the same test. The reservations in this statement should be noted carefully and it should not be concluded hastily that because a new bacterial antigen yields sera with an agglutination titer of only 1 200 it is necessarily a poor and inefficient antigen compared with a virus that gives complement fixation titers of 1 5 000 since obviously we have changed both the specificities and the method of test

In following antibody variation in the course of infection and convalescence it is often satisfactory to follow only the *relative* antibody contents. Nevertheless in comparing new results with those obtained previously particularly in other laboratories it is difficult to be certain that the titration scales are identical unless a common reference serum is available. This applies even when precise physical instruments are used in quantitating the relative titers. For many theoretical studies or whenever a particular reference serum is unlikely to be available to other investigators it is often desirable or even essential that the readings be made or converted into absolute weight units available in every laboratory

Other Precipitation Procedures

The simplest method for demonstrating the presence of antibody consists in carefully layering a fairly concentrated solution of antigen over serum contained in a narrow test tube. The serum being heavier will remain at the bottom and a sharp boundary will be formed. As the two reagents diffuse into one another a thin ring of precipitate will develop in the layer where optimal conditions for precipitation prevail (*ring test*). This procedure is most useful when no data as to the content of antigen or antibody are available and when it is desirable to economize material without wasting dilutions in a possible inhibition zone. It is carried out rapidly and easily but the results are of qualitative significance only. It is nevertheless a valuable tool where only an indication of the presence or the absence of antigen or antibody is required as in tests on supernate

An important modification of this technic has been made recently by Oudin through the addition of a small amount of agar to the serum. Antigen solutions diffusing into this form a distinct and stable band for each component to which there is antibody present. Ouchterlony and Elek have shown that certain advantages result if both the antibody and the antigen are permitted to diffuse into each other and other investigators have devised still further refinements. Although the underlying theory is still being developed agar diffusion methods have already become of considerable importance in serology. Thus it has been possible to demonstrate that some purified and supposedly homogeneous antigen preparations contain at least 7 distinct components and that antibodies corresponding to each exist in the antisera. Details will be found in the review of Oudin (1952) and in the papers of Wilson and Pringle (1955) and Korngold and van Leeuwen (1957)

In a still more extended refinement the technics of electrophoresis and of agar diffusion have been combined in a single procedure. A complex antigen such as horse serum may first be spread out on an agar support by applying an electric potential and the invisible bands each containing one or more horse serum components of a characteristic mobility are then made evident by permitting a suitable rabbit antihorse serum to diffuse into them. The resulting antigen antibody precipitates permit ready visualization of the fact that horse serum contains at least 17 distinct antigenic components—the qualification of a minimum being necessary since we cannot be certain that our antisera contain antibodies against all of the antigens tested. Quite similar results have been obtained with human sera as antigens (Williams and Grabar 1955)

Precipitin Titrations One of the more classic serologic procedures is the precipitin titration already referred to above. As might be surmised this consists essentially in making dilutions of antiserum (generally twofold) and adding to each a constant amount of antigen. The greatest dilution of serum that still gives a visible precipitate is taken as the precipitin titer. Alternatively the amount of antiserum may be kept constant and the dilutions of antigen varied

Examples of the various types of titrations can be seen from Table 9 taken from data of

TABLE 9 TITRATION OF RABBIT ANTIPNEUMOCOCCUS TYPE I ANTISERUM WITH POLYSACCHARIDE ANTIGEN

DILUTION OF SERUM	ANTIGEN CONCENTRATION EXPRESSED IN μg PER ML 256	128	64	32	16	8	4	2	1	0 5	0 25	0 125
1 25	+	+	+	+	+	+	+	+	+	+	+	−
1 50	+	+	+	+	+	+	+	+	+	+	+	−
1 100	+	+	+	+	(+)	+	+	+	+	+	+	−
1 200	−	+	+	+	+	+	+	+	+	+	+	−
1 400	−	−	+	+	+	+	+	+	+	+	+	−
1 800	−	−	−	+	+	+	+	+	+	+	+	−
1 1 600	−	−	−	−	−	+	+	+	+	+	+	−
1 3 200	−	−	−	−	−	−	−	−	+	+	+	−
1 6 400	−	−	−	−	−	−	−	−	−	−	+	−
1 12 800	−	−	−	−	−	−	−	−	−	−	−	−
SUPERNATANT EXAMINATIONS												
1 25	A	A	—	a	a	a	a	a	a	a	a	
1 50	A	A	A	—	a	a	a	a	a	a	a	
1 100	A	A	A	A	(−)	a	a	a	a	a	a	
1 200	A	A	A	A	A	—	a	a	a	a	a	
1 400	A	A	A	A	A	A	—	a	a	a	a	
1 800	A	A	A	A	A	A	A	—	—	a	a	
1 1 600	A	A	A	A	A	A	A	A	—	—	—	
1 3 200	A	A	A	A	A	A	A	A	—	—	—	
1 6 400	A	A	A	A	A	A	A	A	A	—	—	

\+ Precipitate
— No precipitate
A Excess antigen in supernatant
a Excess antibodies in supernatant
() Optimal ratio (fastest reacting mixture)

From D S Martin 1943 A simplified serum dilution method for the quantitative titration of precipitins in a pure antigen antibody system J Lab & Clin Med 8 1477 82

Martin The columns represent titrations in which antibody is varied while antigen is kept constant at the value specified in the heading The endpoints (in terms of serum dilutions) increase as smaller amounts of antigen are employed since inhibition by antigen excess is then avoided However beyond a serum dilution of 1 6 400 (and 0 25 μg antigen) the amounts of precipitate are too small to be detected and no further endpoint can be obtained

In the *horizontal rows the conditions are* reversed (to constant serum and variable antigen) Since the lower endpoints now all occur with 0 25 μg of antigen and are thus independent of the amount of antibody present antigen dilution titrations are quite unsuitable for antibody assay However they can be used for quantitating antigen

The heavy lines in the upper part of the table underscore the tubes that lie in the equivalence zone as determined by the supernate tests If each row of tubes in a titration such as this is observed at frequent intervals after the serum and the antigen are mixed, it is usually noted that one tube begins to cloud first and in time the tubes on either side also evidence signs of increasing precipitation Evidently then, some particular ratio of antibody and antigen will flocculate more rapidly than any other This is often a ratio in the equivalence zone although serologic systems are known that flocculate most rapidly with slight excess of antigen and others of antibody In any case the tube of most rapid flocculation can be used as an experimental indication that a particular ratio has been reached it is not necessary that the value of this ratio be known merely that it be constant for a given system Since for a series of sera of the same specificity the amounts of antigen necessary to give some definite combining ratio with antibody are directly proportional to the relative antibody contents of the sera this *optimal proportions method* can be used to titrate

antibody In the alternative form of the test the antigen is kept constant while antibody is varied The rate of flocculation of antigen antibody complexes is a rather complicated function of all of the conditions and the results of the two types of titrations will not agree unless special precautions are observed (Bowan and Wyman 1953)

Agglutination Reactions

Although most bacterial antigens can be reduced to soluble forms often it is more convenient to work directly with the particulate state For most purposes the antigen may be regarded as a potentially soluble substance which is firmly attached to the bacterial surface by a chemical bond In this state it still can combine with antibody which acts as a bridge linking the organisms together in clumps (*agglutination reaction*) the amount of antibody necessary for this will depend on the serologic system and probably also on the amount and the distribution of the antigen on the particular microbial strain used Thus an agglutinating serum need not give the same titer toward all stocks tested The reaction has the specificity expected of the corresponding precipitin reaction of course many bacteria may also be agglutinated nonspecifically by acids dyes etc A third type of agglutination between certain bacteria and erythrocytes has been studied extensively and a number of diagnostic applications have resulted (Neter 1956)

Titrations involving antibody may be carried out with either living or dead bacteria by diluting out the serum and observing the titer at which the desired degree of agglutination is achieved The reaction is facilitated by heat (37 to 56) and by stirring the subsequent reading is often facilitated if the tubes are first centrifuged lightly The quantitative absolute methods described above for soluble antigens may also be extended to particulate antigens such as micro organisms tissue suspensions or erythrocytes These antigens are added as washed suspensions of known N content and after the reaction the antibody N is determined from the total N recovered as the excess over that added

The agglutination reaction has important diagnostic applications It may be used to identify the serologic types of bacteria isolated from cases or it may be used to detect the presence of antibody in the patient s serum to confirm present or past contact with the etiologic agent as in typhoid fever or brucellosis Where antibodies occur in the absence of acute infection due to previous vaccination or the persistence of antibodies after convalescence estimations of the antibody titer must be made preferably at more than one time to determine whether the titer is rising or falling

Hemagglutination Reactions At least 3 distinct phenomena involving the agglutination of erythrocytes are influenced by antibody and may thus be used to measure the amount of the latter in sera

The first of these discovered independently in 1941 by Hirst and by McClelland and Hare involves the agglutination of erythrocytes by certain viruses The mechanism of the process is far from simple The virus becomes attached to specific receptor sites on the cell surface and then following an enzyme like reaction the virus is again released The initial absorption of the virus can be inhibited by specific antiviral antibody and this may be made the basis for antibody titration (*hemagglutination inhibition test*) The details of the absorption process and of the inhibition test are discussed in the companion volume on viruses

A second hemagglutination reaction involves the adsorption of soluble antigens on erythrocytes and the subsequent agglutination of these coated cells by their reaction with antibody to the absorbed material In general polysaccharide antigens are spontaneously absorbed to cells while protein antigens will absorb only to specially treated so called tanned erythrocytes

A third type of hemagglutination occurs in *immune adherence phenomena which are de*scribed below under complement reactions The receptor sites are distinct from those for virus hemagglutination

Absorption Reactions

If a serum contains antibodies to more than one antigen it may be rendered specific for one of these by removing the others For example bacteria of one general group such as pneumococci usually contain common antigens (C polysaccharides and nucleoproteins) so that organisms of one serologic type will give some reactions with antisera to any of

the other types Therefore if a serum to one type is permitted to react with one or more other serologic types of organisms of that group the common antibodies can be removed and the supernate will contain only antibody for the homologous type specific for the organism originally injected Although absorptions are performed more conveniently with particulate antigens such as bacteria or blood cells they can be made with soluble antigens as well In the latter case however care must be taken that not too large an excess of absorbing antigen is used since it may then begin to inhibit antibody When properly used absorption technics greatly broaden the range of serologic methods both by preventing false readings due to common antigens and by permitting the separation of two cross reactive antigens that could not otherwise be distinguished except by the most refined quantitative methods By their use Irwin (1947) has identified over 20 different antigens in hybrid species of birds

Complement

Complement is the name given to a group of proteins found in normal sera, that participates in a variety of serologic reactions One manifestation of complement activity—the ability of freshly shed blood to resis putrefaction for some time—was noted by John Hunter in 1792 To this is to be added an ever growing list of other activities the hemolysis of antibody coated erythrocytes the lysis of certain sensitized bacteria the capacity to kill sensitized bacteria in the *absence of bacteriolysis the opsonization* of bacteria in the presence of antibody the alteration of the rate of aggregation of antigen antibody complexes and the property of combining with many antigen antibody systems (as in complement fixation) Complement is also involved in the immobilization of spirochetes by specific antibody in the so called immune adherence reaction and in the various manifestations of properdin activity Many of these will be discussed below

It will be evident from the foregoing list that complement—viewed as a single system or reagent—is an important component of many laboratory tests It is also considered to be an important element in the host defense reaction however unlike most antibodies it is present in all normal sera, and the amount of complement present probably varies little with various disease states although its effective result may depend greatly on the simultaneous presence of specific antibodies or other elements that do vary

By the rigid empirical standardization of reaction conditions complement has been employed successfully in diagnostic and other laboratory reactions for more than a half century Nevertheless such common usage has added very little to our knowledge of either the nature of complement or the mechanisms of its actions What little we have in these respects has come almost entirely from a few lines of special investigation much of the recent work being carried out in the laboratories of Ecker Pillemer, Heidelberger and Mayer (Heidelberger and Mayer 1948) At present complement is *again the subject* of active and fertile research and it is possible that a considerable clarification of our knowledge of this complex system may be achieved in the near future

Studies on the chemistry of complement began with the work of Ferrata in 1907 who found that dialysis of guinea pig serum—a prime source of complement—resulted in a soluble and an insoluble fraction When the *latter was redissolved in saline neither it nor* the water soluble supernate from which it had been separated had complement activity Since mixture of the two fractions restored much of the activity it was apparent that complement consisted of at least two components both of which were necessary for it to function Later two other components were discovered one is removed by reaction with yeast cells the other is readily inactivated by primary amines such as ammonia Since the complement activity of whole serum is often abbreviated C the individual components may be referred to as C 1 C 2 C 3 and C 4 (the prime has been agreed upon to avoid confusion with bacterial C substances)

The components differ greatly in their heat stabilities C 1 and C 2 are *relatively heat* labile and their activities are lost by heating for 30 minutes at 50° while the C 3 and C 4 activities are lost only if the temperature is raised to 66° The components also differ in their stability to pH changes and the optimum conditions for preserving one may result in marked losses of others In spite of its relative heat stability C 3 is the first to de

TABLE 10 AVERAGE TITERS OF COMPLEMENT AND COMPONENTS

Source	Whole C Units/Ml	C 1 Units/Ml	C 2 Units/Ml	C 3 Units/Ml	C 4 Units/Ml
Guinea pig	350	2 300	450	3/0	6 000
Human	100	3 /00	170	250	4 000

From Bier O G Leyton G Mayer M M and Heidelberger M 1945 A compa ison of human and guinea pig complements and their component fractions *J Exper Med 81 449 468*

teriorate on standing even at room tempera ture

Purified fractions have been prepared for guinea pig human and pig complements All components appear to be proteins some con tain considerable carbohydrate In guinea pig serum C 1 constitutes 0 6 per cent and C 2 and C 4 0 2 per cent of the total serum pro tein Although C 2 and C 4 activities can be inactivated separately they have not been separated chemically and thus probably are associated with different parts of the same molecule

The hemolysis of antibody coated erythro cytes and possibly most other complement activities require the presence of all 4 com plement components Reagents can be pre pared that lack any selected component and these can be utilized to titrate the activities of the 4 components in any test serum the sera of the various species differ greatly in the activities of their complement components Representative average values found for guinea pig and human sera are given in Table 10

The over all activity of the combination present in whole serum appears to depend largely on the activity of the component pres ent in the lowest titer Guinea pig serum is commonly employed as a complement source for hemolysis since it has the highest over all activity which in turn is due to the relatively high activity of its limiting component C 3 Human complement has less than one third of this over all activity due to a greater limi tation in another component C 2 Cow and pig complements although possessing some components in considerable titer have very low (and hence severely limiting) titers for one or more other components Active syn thetic complements have been prepared with components contributed from 4 species However there are indications that the activities of the various components are not entirely independent of each other as i also evident from the fact that the activity of human com plement is somewhat lower than would be pre dicted from the limiting C 2 titer (Table 10)

Calcium and magnesium ions are also nec essary for complement activity in erythro cyte hemolysis bactericidal action on gram *negative bacteria and possibly other processes* as well Although the stimulatory action of Mg^{++} (in a hemolytic system that probably was not completely deficient in it) was noted by Cernovodeanu and Henri in 1906 the ob servation did not influence complement in vestigators until very recently However these metal ion requirements are now considered of importance In certain titrations either Ca^{++} or Mg^{++} can become limiting through dilu tion of the reagents therefore the endpoint will depend more on this lack than on the amount of antigen or antibody being titrated In other instances the activity of complement —an expensive reagent—may be increased 2 fold or more by providing an excess of the cheap ionic co factors Any reagent that binds Ca^{++} or Mg^{++} will also disturb the tests resulting in one form of ***anticomple mentary activity*** Finally the necessity for the ionic cofactors has stimulated further re search into the possible relation of complement actions to enzyme processes An important lead is the observation that streptokinase activated plasmin may in turn activate the first component of complement to function as an esterase (Ratnoff and Lepow 1957) Some typical reactions involving complement may now be examined in further detail

IMMUNE HEMOLYSIS

Bordet observed in 1909 that if rabbit erythrocytes were injected into guinea pigs the serum of the latter acquired the power of agglutinating and if freshly drawn of lysing the introduced cells The lytic property dis

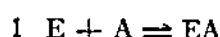
appeared if the serum was heated at from 50° to 60° but could be restored if fresh normal serum was added The heat labile component will be recognized as complement, present in normal as well as immune sera The heat stable component is an antibody resulting from the cellular antigen it is often termed the "immune hemolysin' to distinguish it from the many other natural products that will lyse erythrocytes In a context where the necessity for complement in the reaction is evident, the term is usually shortened merely to hemolysin "

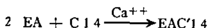
Hemolysins for certain erythrocytes may occur naturally as "normal antibodies' it is not always clear whether these arise from early contact with environmental antigens or are inherited in some manner Hemolysins are also produced by immunization with cell suspensions Rabbit antibodies to sheep erythrocytes are commonly employed in laboratory tests

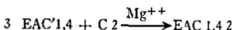
Erythrocytes that have been coated with antibody are termed sensitized cells since they need only the further addition of complement for lysis to begin Sensitized cells are important laboratory reagents, since they act as ready indicators for the presence of complement in active form If sufficient complement is present complete lysis will occur, lesser amounts cause intermediate degrees of lysis In the more quantitative tests the latter are determined from hemoglobin estimates on the supernates With proper co ordinates the normal distribution of these degrees of lysis may be made linear with the amount of complement used and the entire course of hemolysis can be predicted from just two experimental measurements in the appropriate range From such a plot the amount of complement necessary for the lysis of 50 per cent of the cells (50% unit) can be read off In less quantitative titrations a unit may be defined as the amount of complement just sufficient for complete lysis as estimated by eye The amount of complement necessary for a given degree of lysis will depend on the amount of hemolysin in the system, and within limits is inversely proportional to it The most reproducible titrations are made with an excess of hemolysin

As few as 50 molecules of rabbit antibody need be attached to a sheep erythrocyte for effective sensitization and these occupy less than 1 per cent of the cell surface There is kinetic evidence that after lysis of a cell the antibody is released for attachment to another cell, while the complement remains bound and hence unavailable for further reaction

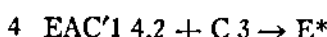
Mayer, Levine and their colleagues (Mayer et al , 1954) have analyzed the hemolytic reaction into some 5 steps

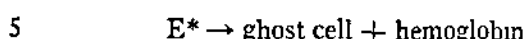

1 $E + A \rightleftharpoons EA$


2 $EA + C\,1\,4 \xrightarrow{Ca^{++}} EAC'1\,4$


3 $EAC'1,4 + C\,2 \xrightarrow{Mg^{++}} EAC\,1,4\,2$


3a *Inactive product*


4 $EAC'1\,4,2 + C\,3 \rightarrow E^*$

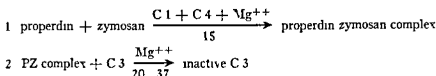

5 $E^* \rightarrow$ ghost cell + hemoglobin

In these steps E = the erythrocyte A = hemolysin C'1 2,3 and 4 the individual complement components and E* the damaged cell that undergoes further transformation Although the whole reaction is normally carried on at 37° Step 2 can take place at 0° if the ionic cofactors are present Due to its high temperature coefficient Step 3 effectively does not take place at low temperatures The hemolytic process can be inhibited by diisopropyl fluorophosphate a known inhibitor of esterases (Levine, 1955)

The Properdin System In 1954 Pillemer and his colleagues announced the isolation from normal serum of a novel protein—properdin—which they suggested played a significant role in host defense reactions Some of the biologic properties of this material have been described earlier (pp 111 112)

Properdin is stated to be a euglobulin with isoelectric point between pH 5 5 and 5 8, and to contain lipid carbohydrate and phosphorus It occurs in Cohn serum fraction III 1 and may be isolated from serum by adsorption to zymosan (an insoluble yeast cell wall carbohydrate), and subsequent elution Purified properdin has a sedimentation constant between S24 and S30 and thus presumably a molecular weight greater than 1 000 000 On standing at 1° C properdin dissociates into a series of molecules with sedimentation constants of 18, 12, 9, 6 and 3 its characteristic activities are lost at the latter stage (Pillemer 1956)

Properdin activity may be recognized experimentally by the following reactions



$$1 \quad \text{properdin} + \text{zymosan} \xrightarrow[15]{C\,1 + C\,4 + Mg^{++}} \text{properdin zymosan complex}$$

$$2 \quad \text{PZ complex} + C\,3 \xrightarrow[20 \quad 37]{Mg^{++}} \text{inactive } C\,3$$

The first stage proceeds significantly only above 15 C while C 1 and C 4 are required initially the end result is the specific inactivation of C 3 In addition to zymosan a variety of polysaccharides from bacteria and from mammalian tissues also react with properdin under similar conditions with the inactivation of C 3 Such substances when they occur in cell walls may represent the substrates through which the bactericidal action of properdin is effected

Pillemer defined the properdin unit as the quantity that in the presence of an optimal amount of zymosan would inactivate 120 ± 30 units of C 3 in 1 hr at 37 C It corresponds to about 0 5 μg properdin N Normal human serum contains not more than 0 02 per cent of its protein as properdin or 4 to 8 units per ml

Preliminary studies have shown that the serum properdin levels remain normal and relatively constant in a variety of infections and also in conditions such as agammaglobulinemia (p 138) diabetes and nephrosis (Hinz 1956) Three exceptions are of interest The first is the fall and then subsequent rise (over a period of a week) in certain cases of pneumococcal pneumonia and of meningococcemia The second is the fall in properdin level following total body irradiation (Ross 1956) A third is the 2 fold to 3 fold rise in the serum properdin level elicited in animals by injection of bacterial lipopolysaccharides (Landy 1956)

The so-called *properdin system* consists of properdin all 4 components of complement and Mg^{++} Wardlaw and Pillemer (1956) have shown that the system is bactericidal to many gram negative bacteria even in the presumed absence of specific antibodies and concluded that this property is an important element in normal or nonspecific defense It is of interest however that one third of the bacterial strains tested appeared to be resistant to this bactericidal action although other strains in each species were susceptible

Properdin differs from antibody in a number of respects the narrow temperature requirement for the reaction of properdin with polysaccharides its presence even in the serum of germ free rats (p 111) its broad specificity its relative lack of variation with time or disease and finally Pillemer notes the specific inactivation of C 3 by the properdin system Nelson (1956a) has advanced evidence that the last is only illusionary and that in reality all components of complement are removed essentially equally Since C 1 2 and 4 are present initially in relative excess their percentage (although not their absolute) reductions are small compared with that of C 3 and this has led to the qualitative conclusion that only the C 3 is affected On this basis Nelson has offered an alternative explanation of the properdin system in terms of the classic variety of antibody acting in conjunction with complement components and thus without invoking any new substance He has obtained evidence for the existence in normal human sera of small quantities of material that appears to be antibody reactive with zymosan as well as related polysaccharides From these observations has evolved the concept that normal serum contains antibodies that cross react with a variety of polysaccharides which have a broad distribution in nature Since many of the reactions in the properdin system represent cross reactions the role of complement is visualized as that of strengthening the bond between antigen and antibody of low avidity

Complement Fixation

Complement is taken up by specific precipitates a property utilized by Bordet and Gengou in 1901 for the development of diagnostic tests The principle is quite simple Suppose a solution of a known antigen A and an antiserum is examined for anti A The two are permitted to react in the presence of complement

If there is actually antibody to A in the serum its reaction with antigen A will bind to it some or all of the complement present This removal of complement is of course not evident to the eye but it may easily be confirmed by adding an indicator system—sensitized erythrocytes—and incubating at 37 C for a short time No lysis should result

On the other hand, if our "unknown" did not contain anti A, no antigen antibody re action can take place, and the complement retains full activity As a result the indicator will be rapidly lysed Although the comple ment fixation reaction is generally used to de tect the presence of antibody, it works equally well in reverse If a known antibody is sup plied it will detect the presence of antigen The reactions of the indicator system remain as in the previous example no lysis denotes the absence of complement, and this can only result (if the system has been properly set up) when an antigen antibody reaction has oc curred Since one of the components for the latter was supplied as a reagent, the other—the antigen—must have been present in the test unknown The complement fixation test differs from most other analytical procedures and therefore is possibly confusing to the be ginner in that a negative reading reaction (no erythrocyte lysis) occurs only when the re action stage (antigen antibody combination) is highly positive Since it is only the latter stage in which we are really interested, such a test would be reported as positive i e com plement was fixed

Since the complement fixation reaction is given by nearly all antigen antibody systems and is quite sensitive it has been widely used in serology A number of experimental modi fications are in vogue these differ chiefly in the amounts (units) of complement employed and in the methods of reading the endpoint (Rice 1948) A refined method applicable to routine diagnosis has been described by Osler Strauss and Mayer (1952) Strict attention to detail is necessary in any complement fixation test all reagents must be controlled and, in particular it must be ensured that neither the antigen nor the antibody separately fix com plement by anticomplementary action at the dilutions used

For more exacting research demands alter native procedures may be used These employ a large excess of complement the variable amounts of complement fixed under the dif ferent conditions are then determined from a series of precise complement titrations with instrumental methods (Osler and Hill 1955 Mayer et al 1948)

Special Tests for Syphilis are discussed in Chapter 26

Bactericidal Reactions

In 1894 Pfeiffer observed that living cholera vibrios injected into a guinea pig previously infected with these organisms showed marked morphologic changes and soon disappeared by lysis The reaction can be reproduced in vitro if antibody complement Mg^{++} and Ca^{++} are present Only gram negative bacteria are susceptible to this bactericidal or additional bacteriolytic, action Since the latter may be difficult to demonstrate or may not occur under the conditions set the reaction is most easily followed by viability tests such as plating or turbidimetric growth assay In the latter form the bactericidal reaction is an ex ceedingly delicate test for antibody the end point amounts of rabbit antibody against *S typhosa* being for example of the order of 0 001 μg antibody N and potent sera may be diluted 1 30 000 to 1 100 000 and still yield titration estimates reproducible to ± 10 per cent (Muschel and Treffers 1956) It is of interest that in the rabbit sera tested the ratio of the bactericidal titers to the agglutinin titers averaged 70, while for human sera against *S typhosa* this ratio was only 2 4 illustrating again that relative titers for dif ferent reaction processes have no fixed pre dictable relation particularly when the serum species is varied

The quantitative properties of the bacteri cidal reaction parallel those of the hemolytic reaction It can be calculated that some 700 to 800 molecules of rabbit antibody are re quired for the endpoint action on one cell of *S typhosa* compared with 400 to 600 mole cules for the corresponding endpoint action of hemolysin on a sheep erythrocyte Substantial rises (and later falls) in serum bactericidal titers against *S typhosa* can be demonstrated during the course of the disease or following vaccination but critical data on the role of bactericidal antibody in resistance to typhoid or any other disease are still to be obtained In in vitro tests bactericidal antibody and a suitable antibiotic such as chloramphenicol when present together exert joint but inde pendent actions on the bacterial culture so that the resultant effect is quantitatively pre dictable from a knowledge of the separate actions (Treffers and Muschel 1954)

Immune Adherence Phenomenon In the presence of their specific antibodies and also

complement bacteria viruses and Treponema pallidum will adhere to human and other primate erythrocytes This immune adherence may be utilized as a sensitive test for antibody (Table 8) with an endpoint visibly determined by the appearance of a particular agglutination pattern in a titration series or by microscopic examination This reaction is to be differentiated from the more familiar nonspecific agglutination of erythrocytes by viruses since the latter reaction does not require complement and moreover is inhibited by antibody (p 123)

Although significant phagocytosis may occur if staphylococci antibody and complement are incubated together Nelson has shown that the *rate is markedly increased if erythrocytes are* also present Motion pictures of the process readily reveal the manner in which the phagocytic cell can strip off bacteria specifically adherent to the erythrocyte without injury to the latter The presence of erythrocytes or other particles is thus an important variable to be watched in in vitro measurements of phagocytosis Nelson (1956b) has advanced the view that such temporary adherence may also be an important element in in vivo phago*cytosis and hence host defense*

Other Properties of Precipitating Systems

The Danysz Phenomenon The data of Figure 16 were obtained by adding to a constant amount of serum the amounts of antigen indicated by the third column of Tables 6 and 7 Quite different amounts of precipitate are obtained if the antigen is not added to each tube in single portions but in successive small in*crements totaling these amounts Due to the* higher initial combining proportions of antigen with larger excesses of antibody and the fact that some time—often days—is necessary for antigen antibody reactions to reverse more antibody is brought down initially by serial additions of antigen and hence less of the latter will be needed to reach the (temporary) equivalence ratio If sufficient time is allowed the system will rearrange to the composition obtained by the one step addition

Incomplete Antibodies Although the mechanism of the process is quite unrelated to the above which occurs due to the variable combining properties of even homogeneous antibody serial precipitation experiments often reveal the presence of another and more specialized form of antibody Because it does not precipitate directly with antigen unless more active forms of antibody are also present this new type of antibody has been termed soluble incomplete or univalent antibody The last term appears to be unjustified unless concrete evidence concerning the exact valence or antigen binding capacity is present in each instance (Kabat in Pappenheimer 1953)

The presence of incomplete antibodies is indicated when the total antibody precipitable by serial addition of antigen is less than that precipitated by the one step addition of antigen In the competitive demand for antigen set up by serial precipitation the normal form of antibody wins out and eventually is removed before little if any of the incomplete antibody co precipitates The latter cannot be detected in the supernates by addition of antigen however it can be co precipitated if added to a sufficient amount of normal antibody and antigen

Serum I (nonflocculating) + 0 10 mg antigen = 0 ppt

Serum II (flocculating) + 0 10 mg antigen = 0 60 mg ppt N

Serum I + II mixed together + 0 10 mg antigen = 1 40 mg ppt N

Serum I contains by difference at least 0 80 mg antibody N

Incomplete antibodies are probably present to some extent in all sera but they are not usually detected except by quantitative experiments performed as outlined or by their ability after the normal precipitating antibody has been removed to block the precipitation of other normal antibody through their antigen binding capacity They may occur as the predominant or even exclusive response in the early stages of immunization (Pappenheimer 1940 Heidelberger Treffers and Mayer 1940) Incomplete antibodies of various sorts have also been found in Rh sensitization (Chap 7) and in some forms of allergy (rev in Grabar 1950) Incomplete antibodies may add directly to insoluble antigens and produce agglutination of the latter or the reaction may be made more sensitive if the coated particles are washed and then reagglutinated with an antiserum to the species in

On the other hand, if our "unknown" did not contain anti A, no antigen antibody reaction can take place, and the complement retains full activity. As a result the indicator will be rapidly lysed. Although the complement fixation reaction is generally used to detect the presence of antibody, it works equally well in reverse. If a known antibody is supplied it will detect the presence of antigen. The reactions of the indicator system remain as in the previous example: no lysis denotes the absence of complement, and this can only result (if the system has been properly set up) when an antigen antibody reaction has occurred. Since one of the components for the latter was supplied as a reagent the other—the antigen—must have been present in the test unknown. The complement fixation test differs from most other analytical procedures and therefore is possibly confusing to the beginner in that a negative reading reaction (no erythrocyte lysis) occurs only when the reaction stage (antigen antibody combination) is highly positive. Since it is only the latter stage in which we are really interested such a test would be reported as positive, i.e. complement was fixed.

Since the complement fixation reaction is given by nearly all antigen antibody systems and is quite sensitive, it has been widely used in serology. A number of experimental modifications are in vogue; these differ chiefly in the amounts (units) of complement employed and in the methods of reading the endpoint (Rice 1948). A refined method applicable to routine diagnosis has been described by Osler, Strauss and Mayer (1952). Strict attention to detail is necessary in any complement fixation test: all reagents must be controlled and, in particular, it must be ensured that neither the antigen nor the antibody separately fix complement by anticomplementary action at the dilutions used.

For more exacting research demands alternative procedures may be used. These employ a large excess of complement; the variable amounts of complement fixed under the different conditions are then determined from a series of precise complement titrations with instrumental methods (Osler and Hill 1955; Mayer et al. 1948).

Special Tests for Syphilis are discussed in Chapter 26.

Bactericidal Reactions

In 1894 Pfeiffer observed that living cholera vibrios injected into a guinea pig previously infected with these organisms showed marked morphologic changes and soon disappeared by lysis. The reaction can be reproduced in vitro if antibody, complement, Mg^{++} and Ca^{++} are present. Only gram negative bacteria are susceptible to this bactericidal or additional bacteriolytic, action. Since the latter may be difficult to demonstrate or may not occur under the conditions set, the reaction is most easily followed by viability tests such as plating, or turbidimetric growth assay. In the latter form the bactericidal reaction is an exceedingly delicate test for antibody, the end point amounts of rabbit antibody against *S. typhosa* being, for example, of the order of 0.001 μg antibody N, and potent sera may be diluted 1:30 000 to 1:100 000 and still yield titration estimates reproducible to $\pm$ 10 per cent (Muschel and Treffers 1956). It is of interest that in the rabbit sera tested the ratio of the bactericidal titers to the agglutinin titers averaged 70, while for human sera against *S. typhosa* this ratio was only 2-4, illustrating again that relative titers for different reaction processes have no fixed predictable relation, particularly when the serum species is varied.

The quantitative properties of the bactericidal reaction parallel those of the hemolytic reaction. It can be calculated that some 700 to 800 molecules of rabbit antibody are required for the endpoint action on one cell of *S. typhosa* compared with 400 to 600 molecules for the corresponding endpoint action of hemolysin on a sheep erythrocyte. Substantial rises (and later falls) in serum bactericidal titers against *S. typhosa* can be demonstrated during the course of the disease or following vaccination, but critical data on the role of bactericidal antibody in resistance to typhoid or any other disease are still to be obtained. In in vitro tests bactericidal antibody and a suitable antibiotic, such as chloramphenicol, when present together exert joint but independent actions on the bacterial culture so that the resultant effect is quantitatively predictable from a knowledge of the separate actions (Treffers and Muschel, 1954).

Immune Adherence Phenomenon. In the presence of their specific antibodies and also

Other units such as the serologic flocculation unit L_f and the intracutaneous skin test unit L are also employed in the preliminary standardizations The position of these various units in relation to curves such as those of Figures 16 and 17 are graphically illustrated for the sera of several species by Cohn and Pappenheimer (1949) When diphtheria antitoxins are compared with a single standard reference serum by in vitro flocculation methods as well as by neutralization tests in animals it is found not infrequently that the potencies obtained by the two procedures do not agree due to differences in *avidity* between test and reference sera which differentially affect the tests

ANTIGENS

General Properties

Complete antigens are substances that can elicit the formation of antibodies as well as react with them They are either proteins or polysaccharides although lipids or nucleic acids may occur in complexes with these Antibody production occurs most generally in a species other than the one from which the antigen is derived Erythrocytes constitute a common important exception (Chap 7) and portions of brain lens or other tissues remote from the blood stream may also be antigenic in the homologous species especially if adjuvants are used The latter are substances such as emulsifying agents that often greatly increase the antibody response and hence are widely used in experimental immunizations (Freund 1951)

Many laboratory preparations of bacterial polysaccharides are actively antigenic in man or the mouse but do not produce antibodies in the rabbit although a recent report indicates they may do so if given in minute amounts These partial antigens—termed haptens—can nevertheless react with antibodies such as those produced against the whole bacterium from which they were prepared More degraded antigens are known that do not precipitate with any antiserum although they do retain some antigen specificity since they can inhibit the reaction of the complete antigen with its antibody

The conditions necessary for antigenicity have again become of interest in consequence of recent investigations on possible substitutes for human blood in the therapy of shock One of these dextran is now known to be antigenic in man (Kabat 1956a) The data on another fluid gelatin are conflicting Although it is antigenic in rabbits if certain adjuvants are added it was not found to be antigenic in limited tests on man (Maurer and Lebovitz 1956)

Size Although the ability to inhibit formed antibodies is still present in peptides of M W 600 and to some degree in amino acids the ability to elicit antibodies appears to be confined to molecules of much greater size A M W of 10 000 may be taken provisionally as the lower limit below this the molecules appear to be excreted too rapidly to act as effective antigens There appears to be no upper limit since giant molecules such as hemocyanin or tobacco mosaic virus are effective antigens

Complexity Although the antigenic materials of most frequent practical interest—whole micro organisms—are often employed as if they were single antigens they are of course composed of many independent antigens In the streptococcus alone some 10 or more distinct antigens have been uncovered among which the C M and T surface antigens hyaluronidase and the streptolysin O are best known In addition all micro-organisms contain dozens of metabolically active enzymes which are present in too small amounts (or are not released) so as to be active antigenically without special isolation and concentration procedures

Protein Antigens

Proteins are found everywhere in biologic material not only as structural constituents but as enzymes hormones etc Their general serologic behavior has been reviewed by Landsteiner (1945) Kabat (1943) and Treffers (1944) More recent studies have been well summarized by Cushing and Campbell (1957)

Bacterial Proteins Although many of the type specific and group-specific antigens of bacteria are polysaccharides this is not invariably the case Many important pathogens including the streptococcus the tubercle bacillus and the cholera vibrios contain protein antigens that are reflected in diagnostic tests In addition the typical bacterial exotoxins such as botulinus tetanus diphtheria (responsible for the Schick test) and the erythrogenic

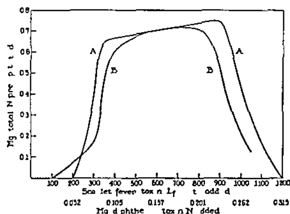


FIG 17 Quantitative toxin antitoxin flocculation reactions Curve A diphtheria system Curve B scarlet fever toxin system (Hottle G A and Pappenheimer A M Jr 1941 A quantitative study of the scarlet fever toxin antitoxin flocculation reaction J Exp Med *74* 545 556)

which the incomplete antibodies were produced This has been applied as the Coombs test in Rh sensitization and to detect antibodies in dysentery, typhoid, brucellosis and Q fever (Coombs and Stoker 1951)

Toxin Antitoxin Reactions In the serologic systems discussed up to this point with the system of Table 6 as a typical example the variable combining proportions of antibody with antigen have resulted in visible precipitation over a considerable range of antigen values A number of other serologic systems of which certain toxin antitoxin reactions are examples exhibit a different behavior in that only one tube in a series may show distinct flocculation while tubes containing one half or twice this amount of antigen show little or no evidence of precipitation even over long periods of time These so called flocculation reactions exhibit inhibition with antibody excess as well as with antigen excess

The flocculation reaction may also be carried out under the conditions necessary for quantitative absolute measurements (Fig 17) In addition to the sharp zones of inhibition on either side, the curves have several other properties that differentiate them from those for the precipitin reaction illustrated in Figure 16 Thus the entire amount of antitoxic antibody in the serum is precipitated at the beginning of the linear portion and the subsequent N increase is due solely to the precipitation of the additional antigen added The situation is therefore like that in the equivalence zone of the precipitation reaction and this permits assay of the antitoxin content of the serum from a single suitable measurement It is also possible to assay directly the fraction of antigen precipitated—a most unusual opportunity for investigating antigenic homogeneity—from the increase in the amount of precipitate produced by two amounts of antigen The production of such flocculating antibody appears to be restricted to the horse, and there, only under certain conditions of immunization with particular antigens These need not be toxic however since flocculating antibody can be produced to egg albumin or to various rabbit globulins

Antitoxin Standardization Although antitoxic antibody may be assayed in weight units, as in Figure 17 the standardization is more commonly done on the basis of arbitrary antitoxin units determined from animal protection experiments To economize on the latter, provisional standardizations are first made with test tube flocculation tests or animal skin tests

Various units have been employed in standardizing toxin and antitoxin For diphtheria toxin the minimal lethal dose (M L D) is the amount of toxin which, injected subcutaneously, will kill a 250 Gm guinea pig in 4 or 5 days The L_0 dose is the amount of toxin that will just neutralize 1 unit of a reference standard antitoxin provided by various government agencies such as the National Institute of Health Due to the difficulties of determining when neutralization is just complete this unit has been largely displaced by the L_+ unit which is defined as the amount of toxin which when mixed with 1 unit of antitoxin and injected subcutaneously, will kill a 250 Gm guinea pig in 4 or 5 days At first sight the L_+ dose might be thought to be the sum of the M L D + L doses but as Ehrlich demonstrated it is considerably greater and the difference between the L_+ and the L doses may be 50 M L D instead of 1 M L D (for specific illustration see Zinsser et al 1939 pp 183 188) This follows from the multiple combining properties of antigen and antibody and from the fact that toxin antitoxin reactions are at least partially reversible, so that when the M L D is added to the neutral L dose part of the added toxin is combined with antitoxin and less than a lethal dose remains free

of specificity Nevertheless if the rabbit antibody to chicken egg albumin described in *Table 6 is tested with the egg albumins of* other species of birds it is found that many of these also react—a phenomenon known as a *cross reaction* Characteristically only a portion of the antibody is precipitable by any cross reactive antigen and this percentage decreases quite generally as the species from which the test antigen was derived differs taxonomically from that of the immunizing *antigen Thus this property may be used as* a semiquantitative guide to degrees of zoologic (and microbial) relationships however if too fine distinctions are attempted formidable difficulties arise

Thus a rabbit antiserum against chicken ovalbumin when tested against other ovalbumins may give amounts of precipitates in this order chicken (homologous antigen) *100* duck 20 turkey 10 guinea hen 5 For another but similarly prepared serum the corresponding values may be 100 10 6 and 2 The reaction patterns for these two sera are similar although the quantitative properties evidently differ Furthermore the percentages obtained with the serum of any animal will vary with the extent of immunization the early bleedings tend to be more specific for the homologous antigen even when allowance is made for their lower antibody content Cross reactivity is also a function of the species of animal immunized thus horse antisera to pneumococcus type III generally give pronounced cross reactions with pneumococcus type VIII while rabbit antisera do so more rarely

If we view these observations for a moment with the sculptor s eye—so to speak—it will be evident that the antibody response does not result in a direct positive to negative replica cast of the entire antigen surface with each feature represented once only On the contrary the response resembles a whole array of incomplete casts each covering only a part of the antigen with extensive duplications of some areas and perhaps the omission of others Moreover no two artisans (our serum ani*mals) give quite the same final product*

This glimpse into the end result of antigen administration is of great interest to the immunologist who must account for the structure of antigens and the details of antibody synthesis He is also forewarned that in the practical problem of proving the identity or *the nonidentity of two microbial or other* antigens exclusive reliance cannot be placed on tests with only a single serum since important elements of the specificities of the antigens may not be represented in the serum sample

Since polysaccharide and protein antigens are constituted from only a relatively limited number of common building units—a few *monosaccharides and a score of amino acids*—their characteristic specificities must result more from the almost infinite number of possible combinations of position order and linkage types As an analogy a house may b constructed of no material not found in neighboring houses and yet exhibit a distinctly individual character

If a sufficiently complete serologic analysis *is made a given antigen derived from a par*ticular species of animal can almost always be uniquely distinguished This point finds application to medicolegal problems in which it may be important to distinguish human from animal blood or in the case of suspected food adulteration beef from horse protein Therefore antigens are said to possess *species specificity* It should not be thought however that all antigens from a given species necessarily have common elements There appears to be no human or bovine element distinguishable for all human or beef proteins Even the albumin and the globulin which *occur together in blood serum are independ*ent in serologic specificity and show no crossing whatever However as we have already seen comparable proteins such as the albumins the hemoglobins and the thyroglobulins from different species often do show cross reactions in some proportion to the degree of zoologic relationships The many enzymes found in a particular micro organism are usually unrelated serologically although considerable crossing may be obtained with comparable enzymes from closely related species

The Structural Basis of Serologic Specificity The early recognition by serologists of the cross reactivities of taxonomically related antigens led to the hypothesis of underlying structural relationships However great technical difficulties—only recently solved even in part—prevented an adequate demonstration of

toxin of the hemolytic streptococcus (responsible for the Dick test) are proteins that have been isolated in purified form Details of their chemical and serologic properties will be found in the book of van Heyningen (1950)

Enzymes All of the enzymes that have been examined have been found to be antigenic and the resulting antibodies will precipitate the enzyme the supernate is therefore devoid of enzymatic activity However the combination of antibody with enzyme need not neutralize the latter and in extreme instances the specific precipitates will retain full activity toward the substrate Mushroom tyrosinase or bacterial catalase provide examples At the other extreme the antitoxin to *Cl welchii* will inhibit completely the lecithinase activity of the α toxin In numerous other cases the enzyme activity can be inhibited to a certain degree only further additions of antibody being without greater effect It would appear from this that the parts of the enzyme molecule responsible for enzyme activity need not coincide (or overlap) with those involved in antigenic (antibody combining) activity Analogous effects have been obtained with toxins from gram negative bacteria and for antibody proteins (in anti antibody studies) in which one biologic property—toxicity or the ability to combine with antigen—is more or less distinct from the ability to combine with antibody Antibodies to intracellular enzymes may neutralize completely the isolated soluble preparation used for the injections but may not inhibit at all the enzyme functioning within the cell presumably because it cannot enter it Antienzymes are proving to be important reagents in studying adaptive enzyme formation and many other problems Their general properties are discussed by Cinader (1957) Sevag (1951) and Marrack (1950)

Polysaccharide Antigens

Polysaccharide antigens may constitute 10 per cent or more of the dry weight of the bacterial cell Among the pneumococci alone nearly 100 serologic types are known of which about one tenth have been characterized chemically There are a number of serologically active polysaccharides that contain only glucose so that structural arrangement is an important element in providing serologic specificity Unlike proteins which occur only in definite and characteristic molecular weights polysaccharides occur over a wide range of sizes The methods of isolation have been reviewed by Burger (1950)

Gram negative bacteria contain O antigens that are extracted in the form of phospholipid protein polysaccharide complexes (Morgan and Synge 1945 Perlman and Goebel 1946) They are responsible for most of the characteristic serologic reactions of this large group and probably are involved in their pathogenic effects as well since protection against some degree of challenge may be secured with antibody directed against them They are responsible for a variety of pharmacologic effects including the local and sometimes systemic reactions that follow TAB vaccination The development of fever in humans after intravenous medication has been shown to be due in some instances to contamination of the solutions with minute amount of gram negative micro organisms and an extensive literature has accumulated on the pyrogenic factors responsible (Bennett and Beeson 1950)

Heterophile or Forssman Antigens Forssman first reported in 1911 that injection of guinea pig organs into rabbits gave rise to antibodies that lysed sheep erythrocytes in the presence of complement This phenomenon depends on cross reactive portions of antigens which are found both in animals and in many micro organisms The term "heterophile antigen now includes all antigens that show cross reactions when the species from which they are derived are too remote for such a reaction to be expected A number of such systems are known Some such as that linking the rhesus monkey and the pig or the horse and the rat are of little general interest while others such as that linking human blood cell antigens to antibodies produced against Pneumococcus type 19 in the horse had—at least in the recent past—definite medical interest since sera with these antibodies could give rise to severe transfusion reactions Heterophile antibodies are found in certain human sera both normally and in higher titer during the course of disease particularly infectious mononucleosis (hence the Paul Bunnell test)

Serologic Specificity

Antigen antibody reactions are rightly stated to be characterized by a high degree

ANTIBODIES

Antibodies are globulins found principally in serum that react with antigens within the limits of serologic specificity The amounts found in serum vary over the lifespan of the individual and are in particular notably influenced by immunization with the conjugate antigen Antibodies of a variety of chemical physical and serologic properties are generally produced even in response to the introduction of a single antigen If complex collections of antigens—such as whole micro-organisms—are used all of these complications are multiplied accordingly

Normal Antibodies It has long been known that both human and animal sera contain small amounts of antibodies against a variety of erythrocytes and micro-organisms Some of these so called normal or natural antibodies such as lysins for particular foreign erythrocytes become evident shortly after birth while other agglutinating or antitoxic antibodies may appear later These antibodies are to be distinguished from the more transient antibodies passively donated from mother to offspring

Although it has be n postulated that the stimulus for synthesizing normal antibodies is also a genetic property it is likely that at least some of the normal antibodies result from contact with environmental antigens as in subclinical infections The scant data available for germ free animals is in agreement with this since these more sheltered animals appear to have significantly lower normal antibody levels The special case of the human isoagglutinins to blood group A and B is excluded from discussion here since no satisfactory theory of their origin has yet b en presented (Chap 7) The existence of normal antibodies is not only of theoretical interest but their possible presence must be taken into account in all experiments employing normal serum

Unity and Diversity of Antibodies All observers agree that the production of antibodies is a complex physiologic response probably of several distinct centers Th physical and serologic properties of the antibodies produced are influenced by a number of factors the animal species injected the nature of the antigen th route of injection and the numb r of injections The amounts of antibody produced are further influenced by the age of the animal and by its nutrition by the dosages of the antigen the spacing of the injections by the physical state of the antigen and by the presence of adjuvants Experimentally it may be modified by hormones such as cortisone or by x irradiation

It was understood by the early immunologists that agglutination could be demonstrated for certain sera that did not give a precipitin reaction and with understandable caution they referred to agglutinins precipitins etc as if these were distinct antibodies By 1930 Zinsser and others realized that differences in titer might be due merely to differences in test sensitivity (as in Table 8) and proposed that the diverse reactions could be due to a single antibody (Kabat in Pappenheimer 1953) Later proof came with the demonstration that purified antibody solutions i olated as precipitins would also agglutinate fix complement and protect mice against infection Today no claim for an atypical antibody of limited serologic activity can be accepted without evidence that there is enough of it present to give the other serologic reactions if it were capable of it

The existence of cross reactions is of course direct evidence that antibodies of different serologic properties may be produced in response to a single antigen These cannot always be otherwise distinguished by their physical or chemical properties However as we will see shortly antibodies of distinctive physical characteristics may be produced as instanced by the so-called γ and T diphtherial antitoxins in the horse By our Unitarian view we must accept as a working hypothesis that each component of such multiple antibody responses may display all of the appropriate serologic reactions

Finally we must again note the existence of antibodies of undoubted limited serologic activity such as those revealed in serial absorption studies (p 129)

Other examples have been provided from the field of allergy Thus Cooke Loveless and others have described for human allergic sera both skin sensitizing and thermostable blocking antibodies The *former*—the so-called atopic reagins —have not been thought to sensitize guinea pigs to anaphylactic shock Kuhns and Pappenheimer (1952) have described a nonprecipitating diphtheria antitoxin that also sensitizes skin although it does not

this in chemical terms In the interim serologists tended to utilize the concept of a serologic unit, by which was meant a distinct activity that could be recognized by serologic means and presumably corresponded to a definite (if unknown) structural unit The experimental problem then became that of determining how small (or large) a molecular configuration would have to be to serve as a serologic unit

Landsteiner provided an approach to this problem in a masterly series of researches extending over a quarter of a century (Landsteiner, 1945) It was based on the fact that although natural antigens could not yet be analyzed structurally they could be modified chemically in known ways and the subsequent serologic effects of these could be determined Thus a tremendous variety of simple chemical substances could be linked to protein by the diazo or other suitable reaction and antibody produced to the modified protein A portion of this antibody was specific for the introduced grouping and did not react with the unmodified protein This specificity could be further explored by tests utilizing chemical substances more or less related to the original one such as optical isomers or for aromatic ring compounds the ortho meta or para isomers In particular Landsteiner could illustrate the dominant role of polar groupings such as —COOH or for amino acids the importance of their number and position in the peptide chain (Landsteiner 1945 Pardee and Pauling 1949)

While the technics used did not permit direct *chemical dissection of protein and polysaccharide antigens* the results gained left little doubt that their structure when known in detail would prove to be sufficiently varied to meet the requirements for serologic specificity In more recent studies employing radioactive tracers attempts have been made to determine how many such serologic units could be detected in various antigens (Francis Mulligan and Wormall 1955)

A second line of investigation utilizes various natural and synthetic products such as polyglucoses or dextrans for which some structural information is available From the extents of cross precipitation or inhibition obtained with these important conclusions can be reached concerning the specificities of both the antigens and of the antibodies produced against them (Kabat, 1956a Heidelberger Bjorklund and Larner, 1957) Valuable information is also gained from degradation studies Thus, human serum albumin gives only a single line in the agar gel reaction after treatment with an enzyme from rabbit spleen this antigen gives 3 distinct lines and thus presumably contains at least 3 serologic units (Lepresle 1955) Although removal of 6 amino acids from chicken ovalbumin results in a significant decrease in its precipitating power several amino acids may be removed from bovine serum albumin without any detectable serologic change (White Shields and Robbins 1955) This latter observation is of special interest in illustrating that even undoubted differences in structure may not result in differences in antibody response detectable by our most sensitive quantitative methods

Final resolution of the problems of serologic specificity will come only when technical methods permit establishment of all of the details of the structure of natural antigens For the pneumococcal polysaccharides we have adequate linkage data for only one system (pneumococcus types III and VIII) and data on the constituent units of some 9 other types Application of this information to the problem of serologic specificity has been made by Heidelberger Kabat and Mayer (1942) and Heidelberger (1956) Other valuable data have come from the study of blood group polysaccharides (Kabat 1956b)

Notable progress has been made in recent years in methods for establishing the amino *acid orders of proteins—an important step in* the analysis of their total structure (rev by Steenberg and Mihalyi, 1957) Thus Sanger has examined insulins from 5 species cow pig sheep horse and whale This protein is composed of an A chain (21 amino acids) and a B chain (30 amino acids) However the amino acid differences among the 5 species appear only in the identity and the order of the constituents in positions 8 9 and 10 on the A chain! (Harris et al 1956) Similarly Li has shown that the principal differences between sheep and hog corticotropin are confined to amino acid positions 25 to 28 and positions 31 and 32 The amino acids above position 24 are believed not to be essential for the hormonal activity of these proteins

few reports of differences among antibodies all need reinvestigation with more modern procedures

This has been done by Porter and by E L Smith and his colleagues for the amino acid composition of certain rabbit and horse antibodies Rabbit antibodies to 4 pneumococcus types were found to be identical in amino acid composition within the experimental error of 5 per cent a common terminal alanine group was found for 8 such antibodies and for normal γ globulin For 4 of these antipneumococcal antibodies and for antiovalbumin the terminal sequence has been further identified to the tetrapeptide stage as alanyl leucyl valyl aspartyl (McFadden and Smith 1955a) The horse antibodies investigated appeared to consist of a mixture of amino acid chains (McFadden and Smith 1955b) In view of what we have noted above in discussing the species specificities of insulin and corticotropin a more complete analysis of amino acid orders may have to be made before any differences will emerge and it is of course entirely possible that the antibody characteristics arise from folding arrangements or other variations other than amino acid orders Nevertheless the present information is a valuable start on a classic serologic problem

Physical Properties A primary classification by physical properties may be effected by electrophoresis which divides molecules according to their electric charge and by ultracentrifugation which separates molecules of different sedimentation constant and thus reflects the molecular weight and shape Descriptions of these procedures will be found in Kabat and Mayer (1948) Longsworth (1952) and Pickels (1952) A typical electrophoretic pattern for plasma is illustrated in Figure 18 The most rapidly moving component is the albumin the globulins decrease in mobility in the order α β and γ By utilizing other buffers or adding antibody against the serum of the species being examined as in immune electrophoresis (p 121) additional components may often be distinguished Most antibodies are associated with one of the γ-components

When examined in the ultracentrifuge antibodies fall into two principal classes one of molecular weight 150 000 to 200 000 characteristic of most human monkey rabbit and some horse antibodies and a second class of MW around 900 000 that includes cow pig and certain human horse and rabbit antibodies

A fraction that is homogeneous by electrophoresis may still yield two or more fractions when examined in the ultracentrifuge and in any case only a portion of any electrophoretic or ultracentrifugal fraction from serum consists of identifiable antibodies the remainder being designated as normal globulin The physical properties of representative normal and antibody components are summarized in Table 11

The complexities of the antibody response are evident the first few injections of horses with diphtheria toxoid often results in antibody associated exclusively with the γ fraction but on continued injection the additional antibody now appears in a new more rapidly moving T fraction in rabbits the hemolysin produced is heavy if the antigen is the sheep erythrocyte and light when human erythrocytes are used The physiologic bases of these and numerous similar observations are not yet known

Although small quantities of antibody may be separated in the standard electrophoretic procedure and still larger amounts are secured in the modification known as convection electrophoresis commercial quantities of concentrated serum fractions are best isolated by the solvent temperature fractionation procedures devised by Edwin Cohn and his group When applied to human plasma these may be adjusted so as to concentrate antibodies to mumps and influenza viruses staphylococcal toxin and the H antigen of *S typhosa* in their fraction II while the antibody to the typhoid O antigen and all of the isoagglutinins appear in fraction III 1 Such fractions which may contain a 20-fold or greater concentration of antibodies over the initial plasma form the basis for commercial γ globulin which has proved to be useful in the prophylaxis and the therapy of certain diseases Cole and Favour (1955) have employed Cohn s methods to fractionate the sera of guinea pigs sensitized to tubercle bacilli Two fractions are of special interest Fraction II (largely γ globulin) contains the antibody to the tuberculopolysaccharide and will passively transfer to guinea pigs both systemic anaphylactic and the immediate type skin reactivity to the polysac

give a Danysz effect or readily fix complement, and it can only co precipitate with antigen However it does sensitize guinea pigs to anaphylactic shock Since this reaction requires 30 to 100 μg antibody N and the sera of allergic patients may contain less than 0 03 μg antibody N per ml the failure of previous investigators to obtain sensitization of guinea pigs with the usual serum dosages may have a quantitative rather than a qualitative basis

The majority of serologic studies have dealt with antibodies as if they were confined to the serum or if found elsewhere (as in lymph) had migrated from serum However in a number of studies antibodies have been reported to occur earlier in the feces than in the serum and from this time course and other evidence it has been concluded that the fecal antibodies arise independently of the serum antibodies (Koshland 1953)

Chemical Properties Information on the chemical properties of antibodies was first secured by subjecting whole serum to the action of heat or various reagents and noting the residual serologic effects Since several groups of antibodies of differing sedimentation constants electrophoretic mobilities and solubilities exist these together with related normal globulins may be isolated on the basis of such characteristics (Isliker 1957) Alternatively, the antibodies with a common serologic specificity may be isolated by precipitation with antigen, irrespective of their diverse physical properties The specific precipitates may be analyzed directly (with corrections made for the antigen contained within), or purified antibody solutions may be prepared for analysis from them Since antibodies are proteins they like all other proteins may be utilized as antigens for the immunization of heterologous species and their serologic (i e antigenic) properties compared (Treffers 1944)

All of this evidence indicates that antibodies can be grouped on the basis of physical and chemical properties and that within such groups the antibodies can be distinguished from each other (and from normal globulins for which no antibody functions have as yet been found) only by their secondary—but characteristically individual—reactivities with particular antigens As might be expected from the common group properties these individual antibody conferring properties must be rather subtile in fact they have eluded our present technical methods for recognizing them The

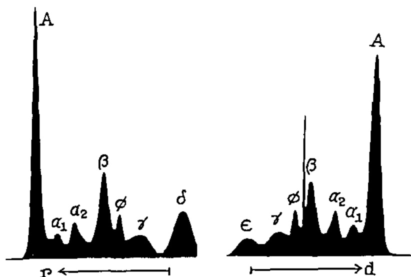


FIG 18 A typical electrophoretic pattern for plasma Patterns are obtained for both the rising and the descending cells arms these may differ due to buffer and protein interactions Albumin is the most rapidly moving component γ globulin the slowest The δ and ϵ boundaries are immobile and represent buffer gradients The corresponding patterns for serum lack the fibrinogen component (ϕ) (Longsworth 1952)

normal) detectable only by sensitive serologic methods in which this protein is used as antigen against appropriate rabbit sera

Hypogammaglobulinemia appears to be a disorder of the hematopoetic reticulum (mesenchyme) expressed in part as a failure of plasma cell formation This is particularly striking after attempts at intensive antigenic stimulation when only normal subjects show a marked increase in plasma cell output that parallels the appearance of antibody Since passively introduced antibody persists at least as long as in normal individuals the disease is not characterized by an unusually rapid and specific destruction of γ globulin It may occur as a sex linked trait affecting only males and beginning early in life or alternatively it may appear at any age in previously healthy individuals of either sex Its modern recognition undoubtedly depends on the fact that the affected individuals would not have survived repeated attacks of infection previous to specific chemotherapy

Hypogammaglobulinemia offers unique opportunities for investigating a variety of immunologic problems (Good and Varco 1955) The lack of resistance to the pneumococcus or the streptococcus correlates well with our ideas on the importance of antibody in these infections Tuberculosis may constitute an important special case Although much remains to be investigated it appears at present that individuals with hypogammaglobulinemia may contract tuberculosis but the incidence and the course of the disease are not unduly abnormal Positive skin reactions to both OT and PPD have been reported (Zinneman and Hall 1956) these were accounted for on the basis of Cole and Favour s observation that in the guinea pig at least the necessary antibodies are in the α rather than in the γ globulin fraction These findings and interpretations are not universally accepted among TB investigators

Although Bruton s original case and a few others provide some exceptions several observers have commented on the otherwise normal incidence and course in these patients of virus diseases such as measles mumps chickenpox and poliomyelitis As a result provocative questions may be raised concerning the generally accepted role of antibody in resistance to and control of virus diseases At present speculation extends to the (unlikely) possibility that protection may be afforded by amounts of circulating antibody too small to be detected by the most sensitive neutralization tests with whole serum or that appreciable amounts of antibody may be formed but are kept from the circulation by firm union with cells

In the investigation of Good and Varco their patients reacted positively to both the Schick and the Dick toxins as might be expected from the absence of antibody An interesting transient exception was afforded by an infant who reacted negatively to both toxins when tested at 4 months of age but positively at 6 months and thereafter when the antibody passively contributed by his mother (known to be a negative reactor) had disappeared Attempts to demonstrate delayed hypersensitivity reactions to tuberculin or to streptococcal antigens gave equivocal to negative reactions in all patients

In a revealing experiment washed leukocytes from normal donors under active immunization with typhoid vaccine were injected into 2 patients with hypogammaglobulinemia Serial bleedings over a 3 week period failed to reveal evidence of the release of circulating antibodies in the recipients even after secondary stimulation of the latter with antigen However skin tests with streptococcal antigens were now for the first time positive for delayed reactions—a result of cell transfer from the positive donors Of marked interest too is the report of a successful skin homotransplant from a normal donor (female age 45 blood group A) to a hypogammaglobulinemic recipient (male age 7 blood group O) This graft was surviving without reaction 8 months after application a reciprocal graft from this recipient to a normal child became necrotic within a month of transfer (Good and Varco 1955) The more general implications of these two types of experiments are discussed below

The Immunization Process

Although many millions of humans as well as countless animals have been injected with various antigens and numerous important observations have been made neither the overall physiology nor the biochemical details are well understood

TABLE 11 PHYSICAL PROPERTIES OF ANTIBODIES AND OF SOME NORMAL GLOBULINS IN THE SERA OF VARIOUS SPECIES

SPECIES	PROTEIN	ELECTROPHORETIC FRACTION	SEDIMENTATION CONSTANT	MOLECULAR WEIGHT*
			Svedberg units	
Cow	Pn antibody†	[illegible]	18	910 000
Pig	Pn antibody†	γ	18	930 000
Rabbit	Normal globulin	γ	7	150 000
	Pn antibody† } Antibody to egg albumin }	γ	[illegible]	157 000
	Sheep cell hemolysin		19	
Horse	Normal globulin		Mainly 7, Some 18	165 000
	Pn antibody†	γ or β = T	18	920 000
	Diphtheria antitoxin	β = T	[illegible] 2	184 000
Human	Normal globulin	γ	[illegible]	156 000
	Pn antibody†	γ	[illegible]	195 000
	Isoagglutinins	$\beta = \gamma_1$	18	
	Wassermann antibody	$\beta_2 = \gamma_1$	7, 19	

* *The molecular weight is obtained from a combination of the sedimentation constant and the diffusion constant which may vary independently*

† Pn antibody = antibody to the pneumococcus specific polysaccharide

charide Fraction IV 10 (largely α globulin) contains antibody to the tuberculoprotein and will passively transfer to normal guinea pigs *a delayed type skin sensitivity to tuberculin* PPD that is maximal at 18 to 30 hours The failure of previous attempts to demonstrate the latter transfer which was successful only if cells were used is explained by Cole and Favour s finding that the activity of fraction IV 10 is inhibited if fraction II is combined *with it as it would normally be in whole* serum (See also Ehrenkranz and Waksman 1956)

Hypogammaglobulinemia (Including Agammaglobulinemia) In 1952 Bruton reported the case of a young boy who within a 4 year period had had 18 serious infections except for *3* bouts of mumps these were all of bacterial origin Laboratory studies indicated that the patient was unable to form circulating antibodies and electrophoretic examination of the serum revealed no γ globulin pattern By 1956 some 66 similar cases had been reviewed (Good and Mazzitello 1956), but since most of these came from only a few clinics it is likely that the anomaly is actually more widespread

The common features of the more extreme cases are (1) an absence (or a very low level) of γ globulin (2) values for other serum proteins that lie within normal limits and thus differentiate the disease from a general hypoproteinemia of malnutritional or other origin (3) a complete absence of the *antibodies to be expected normally in the* population age group such as antibodies to streptolysin streptococcal hyaluronidase or desoxyribonuclease cold agglutinins Forssman *antibodies virus neutralizing antibodies* and significantly isoagglutinins (4) equally lacking are antibodies to bacterial and viral infections from which the individual patients have just recovered (5) injection of the subjects with typical bacterial antigens such as diphtheria or tetanus toxoids or pneumococcal polysaccharides a variety of virus and rickettsial antigens or heterologous bloods does not result in measurable antibodies even after secondary stimulation

Although the term agammaglobulinemia is often used for the syndrome the term hypogammaglobulinema appears to be preferable for the more extreme as well as the intermediate cases since serum that reveals no γ fraction on electrophoresis may nevertheless contain small amounts (such as 5% of

TABLE 12 RESPONSE OF HUMAN SUBJECTS TO INJECTIONS OF PNEUMOCOCCUS POLYSACCHARIDES*

(μg antibody N per milliliter of serum 5½ months after injection)

SUBJECT NUMBER	ANTIBODY TO		
	PN TYPE I	PN TYPE II	PN TYPE V
71	5	9 5	2 5
4	3	22	2 8
76	39	10 4	2 3
80	18	4 5	41 5
82	1	12 5	14 2
Average of 20 subjects	8 3	8	6 5

Heidelberger M MacLeod C M Kaiser S J and Robinson, B 1946 Antibody formation in volunteers following injection of pneumococci or their type-specific polysaccharides J Exper Med 83 303 320

secondary phase is taken advantage of in the practice of giving previously immunized subjects booster shots of tetanus toxoid following injury the response in previously unimmunized subjects would be too slow and ineffective to be of value

Individual Variations in Response Experimenters have tended to grade antigens as excellent good or poor depending on the responses obtained with a limited number of animals or humans While it is clear that true differences in antigenicity not dependent on the misinterpretation of titer differences do exist it is also evident that there are large variations in the responses of individual subjects Moreover while some such as hypogammaglobulinemics may be poor general antibody producers other subjects respond indifferently only to certain antigens and excellently to other antigens This effect is illustrated in Table 12 In spite of certain individual reactions the pooled data do not warrant the conclusion that any antigen is superior (or inferior) to the others

Serologic Paralysis Pneumococcal and perhaps some other bacterial polysaccharides are unique in that following their injection into man the resulting antibody level does not usually decline very rapidly moreover in most instances little or no booster response can be elicited In studying this Felton demonstrated that if relatively large amounts of such polysaccharides were injected into mice a complete but quite specific serologic paralysis was obtained These animals produced no circulating antibody corresponding to the pneumococcus type administered nor were they protected against challenge with the living organisms Normal antibody responses against other serologic types could still be secured if the usual small amounts of these types were used for immunization These polysaccharides are only slowly metabolized or eliminated by the host if present in moderate amounts continuously released from a depot they act as an infinite series of minute booster shots (hence the failure of added secondary injections) if present in excess the polysaccharide merely absorbs all of the corresponding antibody from circulation leaving a susceptible subject (Stark 1955)

Toxicity of Antigens Many bacterial antigens have pronounced pharmacologic actions and the typical exotoxins are among the most actively lethal substances known Since the more important toxins (diphtheria tetanus botulinus) are all involved in the pathogenicities of the organisms and the antibodies against them have a protective effect considerable effort has been expended in making antigenic preparations of these specificities This cannot be done directly in practice since the lethal dosages for most animals are fractions of a microgram amounts too small to provide an adequate antigenic stimulus (except if the most painstaking care is taken to build up tolerance very gradually) For many years underneutralized toxin antitoxin mixtures were utilized as antigens Later it was discovered by Ramon and others that the toxic properties of many toxins could be eliminated by chemicals such as formaldehyde without

As we have noted the physical state of the antigen is of some importance Antibodies can be produced to soluble antigens but the response is greater and more sustained if the antigen is *rendered particulate (and thus, perhaps* less soluble and less readily excreted) This may be done by precipitation with alum *or by incorporation into an oil emulsion base* Additional adjuvants such as mycobacteria or staphylococcus toxin may be added if their separate antigenicities do not interfere Antigens so prepared mav provoke local reactions that aid the over all process (Freund 1951) A variety of routes of injection may be utilized (intravenous, subcutaneous intradermal intramuscular etc) as determined by experience with the subject and the antigen The antibody response is also influenced by the amount *of antigen injected*

Stevens (1956) has tabulated the literature data on this and found the response to be expressed by the equation

$$\log Ab = \log K_1 + 1/n \log C$$

Thus the response is not a linear one and depending on the values of the system constants K_1 and n a 10 fold increase in the dosage of antigen C will result in various amounts of antibody (Ab) The latter is usually nearer the square root (a 3 fold increase) than the 10 fold increase expected on a basis of simple proportionality between antigen and antibody Although appreciable amounts of *antibody may result from only a single injection* greater and more uniform responses result from suitably spaced multiple injections Very *intensive courses of injections in animals may* comprise 4 injections per week for many weeks and often may be attended with a significant animal mortality

In human immunization practical considerations usually dictate that not more than one or two secondary injections be used therefore their proper spacing is important In the preintroduction tests of new vaccines various dosages and spacings are usually tested Although the recommended schedule may not be unique or possibly even optimal the physician should not depart from it without some control tests on the results obtained If the antibody level resulting from an immunization course is to be maintained at a satisfactory level one or more additional booster injections of antigen must be made at intervals of 6 months to 3 or more years *depending on the antigen, and on the antibody* level needed for protection Summaries of the principles and the practical details needed by *the physician* for immunization against specific diseases are given in the review of Edsall (1949), in handbooks issued periodically by the U S Public Health Service and by the American Academy of Pediatrics and in reviews in recent volumes of the Annual Reviews of Microbiology and of Medicine

Time Course The time course for the antibody response following the injection of antigen may be divided into 3 phases the latent period the period of antibody increase and *the period of antibody decline Although there* is indirect evidence that antibody may be produced as early as 24 hours following introduc*tion of the antigen it does not appear in th* circulation during the latent period, which may last from 2 to 10 days or more At the end of this period the antibody appears in amounts that increase to a maximum in 2 to 10 weeks Then it declines Jensen determined from studies on children that the antibody decline followed a second order reaction equation the rate of loss being proportional to the antibody concentration at each given time although the proportionally constant was a characteristic of the individual subject In some subjects one third of the antibody remained after 36 months in others it declined far more rapidly Thus there can be no single absolute safe period following vaccination the results must be described statistically as *a distribution (Pappenheimer 1953)*

Secondary or Anamnestic Responses One of the noteworthy features of the immuniza*tion process is that humans or animals* which have received at least one injection of an antigen from then on—even after intervals of *years—respond differently to subsequent in*jections of the same antigen (Burnet and Fenner, 1949) In general the latent period becomes shorter and *the antibody peak is* reached more quickly and assumes a greater height In consequence of the latter antibody tends to persist longer *This anamnestic or* recollection respon e appears to be serologically specific and reports of rises in old antibody levels *following the introduction of* a new and unrelated antigen have not been confirmed The heightened reactivity in the

troduced into several animal species have now led to the important conclusion that if a foreign antigen is introduced into an animal sufficiently early—before the antigen recognition mechanism is established—the newly operative antibody synthesizing mechanism *does not recognize the antigen as foreign* and will accept its persistence (or later introductions of it) without responding by antibody synthesis In other words it has accepted (or adopted) the introduced antigen as a homologous one

Only a few of the many developments of this principle that are of interest can be noted here As is well recognized except in the special instances of human hypogammaglobulinemia skin grafts are normally accepted only when donor and recipient are of identical genetic constitution as in identical twins in all other instances skin grafts are rejected in a short time (7 to 14 days for initial signs) even when the donor is a member of the same strain of animals The novel recent finding is that an animal may be rendered tolerant indefinitely to tissue grafts from the same species by suitable injection not only of skin but also of other cells such as spleen kidney pulp or white blood cells The timing of the tolerance inducing injection is of crucial importance and departures from the optimum are attended with diminishing success Birth as such does not alter the timing schedule and some species have been rendered tolerant several weeks after birth In most other cases however the optimum period lies nearer or wholly within the period of embryonic life Tolerance is limited to components of a definite antigen specificity and does not extend outside of this although multiple separate tolerances presumably may be acquired and each should be discharged separately Much evidence has accumulated that tolerance is due to a failure of the host to initiate synthesis of the necessary antibodies it may be lost or discharged in specific fashion by providing for the necessary antibody synthesis This may be accomplished rapidly through transfer at any time of lymph nodes from a donor that has been actively immunized against the antigen in question or it may be accomplished more slowly by a similar transfer from a normal donor In the latter instance the lymph nodes do not contain pre formed antibody but unlike the cells of the recipient animal they are not tolerant in their reaction toward the antigen and will begin antibody synthesis soon after being transplanted The existence of this effect has been taken as evidence that the foreign antigen in the tolerant *animal continuously releases soluble marker* antigens Although there is indirect evidence to indicate that antibody is the active vehicle in the rejection reaction circulating antibody has not yet been demonstrated in this respect the situation is quite analogous to that obtaining until very recently for the passive transfer of delayed hypersensitivity

The progress in this active field has been admirably summarized by Billingham et al (1956) briefer more accessible reviews are given in the symposia edited by Chase (1957a) Burnet (1956b) and Converse and Rogers 1955 Burnet also makes extensive use of the marker recognition concept in his latest monograph on antibody formation (Burnet 1956a)

The Formation of Antibodies

Experimental studies on the site or sites of antibody production have been vigorously conducted in recent years The principal directions can only be summarized here and the original papers and reviews must be consulted for details Especially recommended as a start are Burnet (1956a) Burnet and Fenner (1949) the symposia edited by Chase (1957b) and by Pappenheimer (1953) and the papers of Good (1956) Taliaferro (1956) and Stavitsky (1957) (See also Symposium 1957)

The fate of injected antigen has long interested investigators on the view that antigen uptake was a necessary first step in antibody synthesis The earliest studies involved histologic observations on the cellular uptake of antigens marked with dyes later work has utilized fluorescent markers C^{14} I^{131} and other radio-marked antigens (Coons 1954) (Taliaferro 1956)

Soluble antigens such as foreign serum proteins or bacterial polysaccharides injected into mice appear rapidly in the connective tissue and in the cells of the reticuloendothelial system particularly the Kupfer cells of the liver the reticulum cells lining the lymphoid and splenic sinuses and in developing lymphocytes

impairing the antigenicity As a result, relatively large and antigenically active amounts of these detoxified toxins, or *toxoids*, may be injected without eliciting any primary toxic reactions although humans and animals may *in time, become hypersensitive even to puri*fied toxoids The toxic somatic lipopolysaccharides of the gram negative bacteria have proved to be refractory to any such treatments and the "kick" of typhoid vaccines is well known If the injections are repeated for several days (a procedure normally done only in animal experimentation) the fever and other untoward responses decrease and finally disappear This tolerance which is readily lost if the injections are suspended is unrelated to antibody formation (Bennett and Beeson 1950)

Combined Antigens When immunization against a number of antigens is contemplated as in pediatric or military practice the considerations of cost, time and assembly of patients warrant the combination of as many antigens as possible into a single injection mixture Many studies have examined the results to be obtained In a classic instance *Hektoen demonstrated antibodies to 32* out of the 35 antigens injected at one time Several combined vaccines such as the one containing pertussis organisms mixed with diphtheria and tetanus toxoids are in extensive use It would appear that although for some preparations higher antibody levels to each component may be reached if these are administered separately on optimal schedules entirely satisfactory "protective levels may be reached with the combination *Nevertheless* from time to time reports have appeared on the competition of antigens simultaneously administered and the depression of the antibody response to one or more components Therefore random combinations should not be administered with*out control studies on the results obtained*

Passive Immunization Antibody may be acquired not only by active manufacture within an individual but also by passive transfer from a donor An important special case is the *transfer* of antibody from mother to offspring either to the fetus *in utero* or after birth via the colostrum

Passive transfer of antibody is still employed in the prophylaxis of diphtheria, tetanus and measles and formerly was utilized extensively in the serum treatment of pneumonia and other diseases now covered by chemotherapy Two cases may be distinguished If the donor and the recipient are of the same species neither an initial hypersensitivity reaction nor subsequent antibody formation is to be expected However after a short initial equilibration period the antibody concentration will decline continuously as the antibody, like the other homologous *serum constituents, is slowly metabolized by* the host If the antibody is derived from a heterologous species several other reactions become possible If antibody to the foreign species already exists in the recipient (as a result of previous contact) immediate anaphylactic reactions, which may be severe or even fatal, are a possibility and appropriate precautions must be taken Even if no such antibodies pre exist the introduced serum proteins will act as antigens and give rise to antibodies that ultimately will eliminate them from the circulation In addition a large percentage of the recipients may respond with serum sickness of varying degrees of severity in from 7 to 10 days following the administration of the new antigen this is due to the reaction of the latter with the newly formed host antibody to it (Raffel 1953)

Immunologic Tolerance

In 1945 Owen described an observation on the blood types of certain cattle twins that has stimulated a considerable amount of exciting recent research of great potential value to medicine and surgery The initial observation was deceptively simple As is well known superfecundation can occur in cattle thus bovine twins may *have separate sires Owen* found that if the sires were of different blood types the twins had a new common type consisting of a mixture of all the combined parental antigens Since only a portion of these cell antigens could be inherited directly *by either twin the* remainder obtained via an initial common circulation must be considered as foreign antigens to that twin and the cells would normally be expected to be eliminated rapidly following antibody formation against them However the mixture of cells persisted throughout these animals lifetimes The challenging problem was why was this so?

Further studies on this system and on analogous effects obtained with a variety of blood cells tissues and soluble antigens, in

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sponse (8) Antigens are first taken up largely by macrophages of the RE system—not by plasma cells or lymphocytes (9) Antibody is probably produced exclusively by cells of the plasma cell lymphocyte series, not by RE cells Cells actively producing antibody show increased RNA in their cytoplasm, it is this that defines the plasma cell (10) Burnet maintains that antibody is or can be produced after all antigen in any recognizable form has been destroyed or eliminated from the body also, that antibody must be produced by descendants of the cells primarily involved (These latter are the two most controversial points) (11) There are hereditable and environmental influences that determine the amount and the kind of antibody produced (12) In addition to the production of classic antibody there are immunologic responses of the 'hay fever" type and the tuberculin" type Are these all produced by one system?

As we have noted in 10 above it is a highly uncertain issue whether or not antigen must always be physically present when antibody is being synthesized On the assumption that it is Mudd Haurowitz and Pauling have attempted independently to explain the function of antigen as *directing the order of amino* acid deposition or the folding of a polypeptide chain in terms of a complementary action with antigen as a direct and necessary template In defense of this general viewpoint we should point out that it is only in those cases in which antigen may reasonably be expected to persist—as evidenced by occasional isolations of living micro organisms in certain latent viral and rickettsial infections 20 to 40 years after the active disease—that truly long lasting antibody is found Burnet while fully aware of this point discounts its pertinence here and has elaborated a more biologic view *in which antigen is* only required at an initial stage, to modify protein synthesizing cells that can then replicate themselves indefinitely in the absence of antigen An attempt to integrate some of these theories and in particular to account for the *difficult problem of the* secondary anamnestic response has been made recently by Stavitsky (1957)

In view of the many gaps in our knowledge the theories of antibody formation must at this stage be more argumentative than expository The immunologist should not be unduly criticized for his vagueness, since even the biochemist has as yet failed to furnish us with a detailed account of the synthesis of any nonantibody protein

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6

The Allergic State

The term *allergy* designates an altered reactivity of the tissues toward particular substances as judged from previous experiences of the same individual or from the experiences of other individuals of the same species [1] This definition must exclude the occasional instances of *idiosyncrasy* in which one encounters an inherent qualitatively abnormal reactivity toward physiologically active material e g morphine The manifestations of allergy in man include pathologic phenomena as diverse as serum sickness food allergy hay fever and asthma infantile eczema poison ivy dermatitis sensitivity to antibiotics and synthetic drugs and sensitivity to products of microbial origin such as tuberculin The inciting substances indeed various in nature induce the altered reactivity following penetration by ingestion absorption through mucous membranes or through skin mechanical injection or invasion of the tissues by pathogenic microbial or viral agents or parasites The existence of an allergic state becomes evident usually only through a new exposure to the same material or one closely related to it chemically but reactions can be ascribed in various instances to vestiges of the inciting agent that remain in the tissues at the time of conversion to the allergic state

Obviously the opportunities for exposure are quite unequal with the different classes of allergens it is evident that many individuals make adequate contact with poison ivy or poison oak while few acquire pollen-oil or tulip-bulb dermatitis In very many cases sensitization will be the result of repeated exposures and the necessary number of these varies among individuals and is indicative of differences in the host factors determining susceptibility to sensitization With respect to sensitization with pollens foods and the like giving rise to the so-called clinical allergies of man (asthma hay fever hives and so on) the genotype of the individual has been said to exert absolute control over the capacity for sensitization and for such individuals the descriptive adjective atopic has been coined However this matter of genotype and exposure has not yet been subjected to adequate examination.

Although the forms of allergy are so various that the reactions clearly belong in different classes it is certain that very many of them are due to the occurrence of *antigen antibody reactions in relation to the tissues* whether organized tissues are involved directly or indirectly through reactions with free cells or platelets In this sense the allergic state will depend upon the presence of antibodies that reflect prior contact of the individual with some particular *allergen* Whenever antibodies can be demonstrated reactions ensue very shortly after application or adequate absorption of the corresponding aller

[1] The term is used here in the original sense of von Pirquet (1906) who introduced it to cover all changes induced in the state of reactivity in consequence of contact with any living thing or inanimate substance

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TABLE 13 TYPES OF ALLERGIC INFLAMMATORY RESPONSES IN MAN*

	IMMEDIATE TYPE RESPONSES		DELAYED-TYPE RESPONSES	
	Wheal and Erythema†	Arthus type Anaphylactic type	Contact Dermatitis	Infectious Allergies
Clinical state	Hay fever asthma Serum sickness Purpura Physical allergies Some infectious allergies	Serum sickness Erythroblastosis Purpura ? Agranulocytosis ? Polyarteritis ?? Rheumatic fever	Cutaneous rashes	Tuberculosis tularemia brucellosis Lymphogranulomatosis smallpox mumps Histoplasmosis and other mycoses Trichinosis
Sensitizing material	Pollen molds ka pok *danders* Antibiotics a few drugs special allergens (ascaris toxoid horse serum etc) Altered tissues of the individual	Soluble proteins *Drugs antibiotics* Polysaccharides of bacteria and parasites Erythrocytes (Rh pos) ?	Poison ivy *Plant oils* Plastics Simple chemical Antibiotics	Bacteria *Viruses* Fungi Parasites
Antibody	Present in serum Nonprecipitable Heat labile	Present in serum Precipitable Heat stable	Absent in serum Absent in cells Unknown	
Transfer of sensitivity	With serum	With serum	Not with serum With cells	
Cytotoxicity of antigen for explanted sensitive cells	None	None	Present	

* Adapted from Lawrence H S 1956 Am J Med 20 428-447

† Urticaria gastro intestinal disturbances angioneurotic edema and eczema although immediate type allergies may not exhibit cutaneous reactivity nor presence of reagins Foodstuffs often represent the offending allergens The position of infantile eczema is undetermined

In our consideration of allergic manifestations principal emphasis will be given to evidences *of the participation of antibodies in* allergic phenomena rather than to a description of allergies as clinical entities and to this end instances of duplication of allergic responses in a normal individual by means of serum or cells taken from a sensitive individual—so called *passive transfer*—will be stressed The allergic individual it should not be forgotten is usually one who has become sensitized *actively* in the immunologic meaning and the intensity of his responses are often greater and more complex than are duplicated through passive transfer of isolated elements With active sensitization there is continued capacity to produce antibody and cellular factors greater reservoirs of these and a probability of ready redistribution

REACTIONS OF IMMEDIATE TYPE

Because of facility in laboratory manipulations the allergic responses of animals have been studied in great detail and afford the bulk of our knowledge concerning antigen antibody reactions in relation to tissues Historically the injection of antigen *into actively* sensitized animals by the intraperitoneal or intravenous routes led to the discovery of *systemic shock* (anaphylaxis) whereas injection just below or into the skin led to the finding of *local tissue damage* (the Arthus reaction) Both lines of study have made uniquely additive contributions

gen consequently, they can be called "imme diate with regard to the time of inception, although several hours may be required for full development Allergic reactions of the so called delayed type are not fitted readily into this scheme Such reactions require at least several hours after introduction of the allergen before an effect is manifest (reactions to poison oak or poison ivy most drug sensitivities reactions to products of micro organisms—tuberculin, mallein, histoplasmin) and show progressive changes for 2 to 3 days or longer These reactions, occurring even in the absence of circulating antibody, are attributable to special properties of the white cells probably lymphocytes Despite the special and unexpected features that are coming to light through the newer attacks on the problem it still appears likely that the cellular mechanism of delayed type allergies is immunologic in nature and is connected with the antibody forming apparatus Such allergic responses are characterized by the known immunologic attributes of *specificity* and are capable of *specific desensitization* It is not unlikely that we have to deal with cell bound antibodies of a special sort perhaps a primitive type of antibody Nevertheless it would require extrapolation of present immunologic knowledge to urge such a ready definition for all allergic reactions as "clinical symptoms conditioned by previous sensitization and mediated by antigen antibody reactions (Halpern) however sound it may prove to be as a working hypothesis

Owing to these differences in mechanism the two chief categories of allergic reactions—the immediate and the delayed types—should be examined separately (Table 13) although in practice they are not always sharply distinguishable In man the two have also been epitomized respectively as the urticarial and the tuberculin" types of reactions Because the relation of antibodies and cells is still rather obscure and it is likely that allergenic stimuli incite both mechanisms in some measure, it seems best not to oversimplify the subject by drawing the sharp line that the words antibody and "cells respectively would imply

A rough outline of the principal classes of reactions in idealized tabular form is provided as a first orientation Systems of this sort require supplementation, particularly with respect to a fuller appreciation of the gamut of sensitizing materials and the variability in occurrence of wheal and erythema and serum antibodies in the immediate type allergies of man

Inspection of the allergenic excitants listed in Table 13, particularly of the nonliving materials reveals that some of the allergenic substances are frank antigens, others (pollen extracts) can be shown to exhibit some degree of antigenicity and many indeed are obviously not antigens in their own right For this reason it may be convenient to use for all of them the term *allergen* or *allergic excitant* So far as experimental analysis has been carried out, it seems probable that many of the nonantigens do combine with proteins or other substances of the host s tissues and assume thereby the nature of an artificial antigen although some alteration in the original structure owing to intermediate metabolism may at times be involved It may be helpful to think of such antigenic complexes presumed or known to arise from the interaction in vivo between chemical and body constituents as *derivative antigens* A special instance is provided in the *physical allergies* in which the allergic insult stems from the action of physical stimuli (e g , exposure to special wave lengths of light or to chilling) on substances of the body forming products that are capable of reaction with the hypersensitivity apparatus

The response of the tissues in allergic episodes may be abrupt owing to the effects produced by liberated physiologically active substances such as histamine and serotonin but the characteristic response is an *allergic inflammation* On the basis of histologic studies of reactions other than the truly evanescent ones Roessle suggested special terms *normergy* for the norm of the inflammatory responses of comparable normal tissues to a given stimulus *hyperergy* for supranormal reactivity *anergy* for lack of normergic reactivity (owing either to existence of an immune state or to abnormal cellular physiology) and *hypoergy* has appeared as well commonly to mean a lessened reactivity subsequent to known sensitivity It will be evident that nearly all of the manifestations of allergy are instances of increased levels of reactivity (*hyperergy*)

of visceral congestion There is a decreased coagulability of the blood

Lesser degrees of anaphylactic sensitivity than the rapidly fatal form described here are to be encountered minimal symptoms include scratching defecation and the characteristic coughs and dyspnea may be transient or not evident

There is another form of shock (protracted shock) in which dyspnea and bronchoconstriction are much less prominent or even absent this results rather unpredictably usually from subcutaneous or intraperitoneal injection of antigen into the sensitized animal or rarely from intravenous injections (Williamson 1936) Instead of shock that is explosive in character there is a profound depression at times comalike and often with copious tearing accompanied by a marked drop in body temperature (as much as 8 or 9 C) the shock can last for several hours before death or recovery occur Indeed the guinea pig that exhibits the protracted form of shock instead of acute death is perhaps more informative one sees more drastic changes in blood pressure which after an initial rise falls steadily during the entire period of shock almost complete loss of coagulability of the blood a leukopenia a marked decrease in numbers of blood platelets and a diminution in the titer of complement (see Chap 5) in the blood owing to fixation by antigen antibody complexes heparin or participation of its components in the activation of plasmin (*vide infra*) When death ensues one observes instead of a ballooning of the lungs edema and hemorrhages of the lungs and congestion of the viscera especially the liver (cf Winter 1945)

In studies of acute shock induced with antigens coupled to radioactive tracers (Warren and Dixon 1948 Dixon and Warren 1950) the shocking antigen was found to localize around the bronchi particularly in the collagenous tissue between the smooth muscle layer and the cartilage rings this would indicate that specific antibody exists in high local concentration a rather unexpected finding Furthermore these workers found evidence when death failed to ensue until the first 2 to 3 minutes had elapsed of the relaxation of smooth muscle occlusion of the bronchi being then ensured by massive interstitial edema Possibly massive edema may play a larger role than was suspected previously

The Mechanism of Anaphylaxis Death from acute shock in the guinea pig is attributable to contraction of the smooth muscle around the secondary and tertiary bronchioles a prominent anatomic feature in this species The air passages are occluded through infolding of the bronchial mucosa and with lungs remaining distended death results by suffocation Contraction of smooth muscle also explains bristling of hair peristalsis and defecation and contraction of the bladder with involuntary micturition (see Organ Anaphylaxis)

The cause for the contraction of smooth muscle for arteriolar and venous spasm (causing the cyanosis) and for the changes in blood pressure is probably the liberation of histamine and serotonin The important event appears to be damage to cells following interaction of antibody with antigen and complement with subsequent events largely determined by materials leaving the damaged cells and by the very fact of tissue damage itself The liberation of heparin determines the decrease in coagulability of the blood and perhaps because of its affinity for prothrombin and complement (literature cited by Gregoire 1946) may even tend somewhat to mitigate shock heparin is inhibitory to the release of histamine and serotonin from platelets by thrombin (Humphrey and Jaques 1955)

Recently it has been emphasized (Benditt and Rowley 1956 Weiser 1957) that mast cells are quite susceptible to damage by reason of antigen antibody reactions liberating not only the physiologically active substance heparin but also histamine and varying with the species 5 hydroxytryptamine (serotonin) Histamine and serotonin are present also in the blood platelets of many species and are liberated upon damage Histamine to which attention was directed particularly because of brilliant studies on anaphylaxis in the dog is also said to arise in the guinea pig from many tissues—aorta lungs uterus seminal vesicles *inter alia* and moreover by simple contact of the isolated perfused sensitive tissues with antigen at body temperature (Bartosch et al 1932 1933 Schild 1939) Choline and acetylcholine are found to be given off during shock by the sensitized guinea pig heart (Went 1936 Farber et al 1944)

While chief attention has been given to the role of histamine—upon injection into normal animals it imitates fairly well the characteristic species peculiarities of anaphylactic shock

ANAPHYLAXIS

Anaphylaxis may be defined primarily as an acute *systemic* reaction, species characteristic that is exhibited by animals in the hypersensitive state *upon reinjection of the same* material these systemic reactions come on soon after the injection and are definitely of the early type [2]

The word anaphylaxis was coined by Richet in 1902 as a term to contrast with prophylaxis in order to describe a state of excessive susceptibility discovered in dogs that he was attempting to immunize to toxic materials Subsequent observations made with guinea pigs by Theobald Smith Otto, Rosenau and Anderson Richard Weil, and many others established the phenomenon as one that resulted from antigen antibody reactions occurring in vivo for most important of all, it was discovered that serum from a guinea pig undergoing active anaphylactic sensitization could sensitize normal guinea pigs passively (*passive transfer*)

For an understanding of the complex events that occur in vivo parallel studies on different species of animals have proved to be most informative A major factor in the analysis of course was provided by the development of Sir Henry Dale s discovery of histamine the first of the physiologically active materials to be recognized Recently the unstable substance serotonin (5 hydroxytryptamine) has attracted attention with regard to its possible role in the mechanism of anaphylactic shock

As a basis for reviewing the anaphylactic *type of responses by man we shall first consider* in some detail anaphylactic sensitization of the guinea pig then the differing information that has been provided by other species

While many aspects of anaphylaxis are shared largely there are special characteristics (Table 14) For example in the guinea pig we have clear evidence of the ostensible fixation' of antibody to tissues with positive responses obtainable months after antibody is no longer detectable in the serum an inhibitory effect of excess circulating antibody and a necessary period for fixation (or equilibration?) of antibody when it is brought to new tissues The rabbit in contrast, shows only slight evidence of the fixation of antibody to tissues—chiefly in the pulmonary arteries and certain other vessels—but requires circulating antibody in excess in order *to undergo anaphylaxis* a major source of physiologically active substances causing the anaphylactic syndrome is apparently the platelets and the white cells From the event of anaphylaxis in the rat and the mouse, a broadened basis has been provided for suspecting the existence of shock mediating substances other than histamine The horse with its more typical distribution of sweat glands shows sweating and urticaria as well as pulmonary effects

Anaphylaxis in the Guinea Pig

The anaphylactic state is readily established in the guinea pig within 10 to 21 days by means of a single injection of soluble foreign protein in the order of 0 1 microgram to 1 mg of crystalline ovalbumin or 0 0001 ml *to 0 01 ml of horse serum preferably injected* into the skin With some materials that are less antigenic than native proteins several preparatory injections may be required Because the guinea pig does not form antibody readily and antigens can remain in excess for some time the use of large amounts of native protein antigens can delay appearance of the anaphylactic state for perhaps 6 weeks or more The symptom complex known as anaphylaxis is demonstrable when a second injection of the antigen (0 1 to 10 mg) is given by the intravenous route in the course of 30 to 60 seconds or alternatively larger amounts by the intraperitoneal route in which case shock often not so acutely manifested starts when sufficient of the antigen has been absorbed

Within the following minute restlessness is evident the hair, especially at the nape bristles often feces and urine are voided the animal scratches at the muzzle with a wiping motion of the forepaws coughs arches its back and raises its head with obvious dyspnea it gives a series of jerks sways goes into violent tonic and clonic convulsions and falls over with evident cyanosis Death ensues after a few shallow gasps All this occurs often within a period of 3 to 5 minutes Postmortem search reveals firm inflated lungs actively beating heart, active peristalsis and evidences

[2] Comprehensive reviews of the subject are presented in Doerr (1950 1951) Seegal 1935) Wilson and Miles (1955) and Kabat and Mayer (1948)

noted by Humphrey and Jaques (1955) the latter property is acquired by normal plasma pretreated with anaphylatoxins such as kaolin

It is interesting to note that the injection *of glycogen will cause sequestration* of white cells and disruption of platelets—features present in anaphylactic shock, but without producing shock (Rocha e Silva 1950)

Organ Anaphylaxis Schultz Dale Test The features of anaphylaxis encountered in the intact sensitized guinea pig are met with also in the individual organs In 1913 Dale demonstrated that the excised uterine tissue of sensitized guinea pigs reacted upon contact with specific antigen (cf Fig *19*) and thereby confirmed the less conclusive report of Schultz (1910) with intestinal segments The uterine tissue reacted even after most of the animal s serum had been removed by perfusion This notable experiment changed the course of thinking at the time since it shifted emphasis away from humoral factors

Upon contact with the specific antigen uterine muscle tissue or intestinal segments contract in the lung either free or in the isolated heart lung preparation of Starling bronchospasm and occluding constriction may *be demonstrated blood vessels exhibit con* striction strips of gallbladder contract the isolated heart exhibits an increased rate of beat arrhythmia and constriction of the coronary arteries (Wilcox and Andrus 1938 Andrus and Wilcox 1939) and the like All these responses are transient the tissue soon returning to the normal state while antigen is still present

The anaphylactic test is often carried out simply by testing isolated uterine strips or intestinal segments By this means one can readily demonstrate the complete loss of anaphylactic (but not physiologic) reactivity of the tissue following one maximal response to specific antigen and through this demonstra*tion to rule out nonspecific causes for shock*

In the Schultz Dale test the tissues (uterine horns of nonpregnant animals of about 250 to 350 Gm weight or segments of intestinal ileum) of guinea pigs that have been sensitized either by a prior injection of antigen or passively by injection of antibody rich serum are su pended in warm oxygenated baths of suitably balanced physiologic solutions lacking carbonic acid carbonate buffer with one end



Fig 19 Schultz Dale Test Key *A* addition of antigen (ovalbumin) to the bath X complete replacement of bath fluid Hist histamine added to secure a concentration of 1 20 000 000

fixed and the other connected with a writing lever When the normal muscular rhythm is established antigen is added to the bath After a latent period of approximately 30 seconds the muscle undergoes a tonic contraction which is registered and magnified by the writing lever the excess antigen is then removed by draining and replacing the bath fluid once again under approximately normal muscular rhythm the tissue makes contact with the same antigen this time without effect (specific desensitization) finally showing that the lack of response is not due to progressive dysfunction of the muscle in artificial environment a test is made with a nearly threshold concentration of histamine (or pilocarpine or acetylcholine or mecholyl [acetyl *beta methylcholine chloride*]) by *which* a typical contraction must be demonstrable (The desensitization of course pertains only to the antibody mechanism for inducing muscle contraction) This series of events is illustrated in Figure 19 which shows the simultaneous contraction of the uterine horns from a sensitized guinea pig mounted individually in the same bath and exposed to specific antigen as indicated

Passive Transfer It was early discovered that serum from a guinea pig undergoing active anaphylactic sensitization could passively sensitize normal guinea pigs the recipient animals exhibiting typical anaphylaxis when an injection of antigen was made within the next few days but not before some hours had elapsed (*vide infra*) The principle has been established not only with homologous antibody that is antibody derived from and

—more recently a role has been seen for serotonin, particularly in the rat and the mouse (Weiser 1957), and a few workers have urged, but not yet convincingly, a role for acetylcholine in anaphylactic shock. There have been several observations pointing against histamine as the sole mediator of shock. The guinea pig uterus, poisoned by histamine so that it responds to further histamine solely by relaxation, nevertheless contracts to addition of specific antigen and to certain agents which produce anaphylactoid responses—peptone (Schild) and snake venoms (Kellaway, 1938). Again a special "slow reacting substance" was detected by Kellaway and Trethewie (1940) in perfusing the lungs of sensitized guinea pigs and Humphrey and Jaques (1954) found an unidentified active substance other than histamine and serotonin in platelets from man, rabbit, guinea pig and ferret. Campbell and Nicoll (1940) and Brocklehurst (1953) have also found evidence that indicates action of material other than histamine.

With regard to tissue damage, injury to the vascular endothelium results in rapid development of edema, a principal lesson from study of the Arthus reaction; perhaps this process accounts also for a temporary attachment of leukocytes and platelets to the endothelium of small vessels with resulting leukopenia. Whether tissue damage arises directly by reason of antigen antibody union in relation to cell surfaces or indirectly through the activation of a proteolytic enzyme of the serum ("plasmin") from its precursor plasminogen is not known (cf. Ungar 1947, Rocha e Silva et al. 1947, Burdon et al. 1951). Apart from activation of the enzyme, a further problem is posed by the presence of at least two types of serum antiproteases that normally serve to inhibit the enzyme. From a study by Norman, 1957, it appears that one inhibitor in serum combines rapidly and dissociably with activated plasmin, the other, permanently but more slowly. The net effect is to permit a definite though temporary proteolytic role to plasmin in the presence of a specific substrate.

A decision as to the role of the various substances in shock is obviously difficult to reach, for minute amounts liberated locally within sensitized tissues may represent effective concentrations but can escape detection in the circulation; this argument is the more valid if antibody is accumulated electively in certain tissues, as the work of Warren and Dixon (1948, 1950) with "tracer" antigens would indicate. Moreover, the occurrence of several simultaneous reactions with different mechanisms seems to be increasingly probable.

Apart from pharmacologically active materials already mentioned, such as histamine and serotonin, evidence for another type of humoral factor recurs repeatedly: the older concept of a toxic substance (Friedberger's "anaphylatoxin") that was believed to arise in vivo by proteolysis (cf. Bronfenbrenner 1915). The recent finding of Germuth and McKinnon (1957) that soluble antigen antibody complexes (formed in antigen excess) induce anaphylactic shock bears on the "anaphylatoxin" concept and perhaps may account for it completely.

Anaphylactoid Reactions. While an antigen antibody interaction is the primary trigger mechanism in systemic anaphylaxis, some of the gross symptoms of anaphylactic shock can be reproduced in normal animals by certain classes of materials; such reactions, termed *anaphylactoid*, have been studied eagerly in the belief that they would help to explain the basic mechanism in anaphylactic shock. The intravenous injection of bacteriologic peptone causes smooth muscle contractions and the characteristic exitus with fully inflated lungs, the mechanism appearing to involve liberation of histamine from the tissues (Dragstedt), and in amounts of the order of magnitude of that set free in anaphylactic shock. The same mechanism is ascribed to the use of crystalline trypsin injected into the intact animal or used for organ perfusion or with platelet suspensions (Rocha e Silva 1941, Humphrey and Jaques 1955) and for snake venoms and staphylococcal toxin used for perfusion of guinea pig lung (Feldberg 1937, 1941).

Anaphylactoid reactions also result from the injection of normal serum that has been incubated with kaolin, barium sulfate, starch, agar and the like, or from reinjection of whole blood in the "preclot stage" (Novy, DeKruif et al. 1917). The effect is sometimes attributed to removal by absorption of antiproteases which usually hold in check the action of serum enzymes on body protein. A close correlation between development of proteolytic activity and the activity of plasma in releasing histamine from rabbit platelets was

considerably when Freund and Whitney (1928), in studies with *rabbits* found that antibody diffuses slowly into the uterus and the skin and is then beyond the reach of perfusion fluid presumably in the intercellular spaces but it is readily recovered when the perfused tissue is ground with sand These workers suggest that the incubation period seen in passive anaphylaxis represents the time necessary for the establishment of an intimate relation between the antibodies and the tissues rather than fixation If so somewhat different sites would be affected in the anaphylactic shock that results from injection of antigen antibody complexes (Germuth) A latent period is to be observed also in Passive Cutaneous Anaphylaxis

Nonspecific Inhibition of Shock A wholly temporary and nonspecific refractoriness can follow the administration of drugs such as anesthetics that depress the animal s physiologic reactions during the combination of antigen and antibody Special mention should be made of adrenalin (epinephrine) which both relaxes smooth muscle and contracts some of the peripheral vessels and is particularly valuable for therapeutic use in human allergies of the immediate type and also the groups of drugs that are termed antihistaminics In adequate dosage the latter can block the appearance of anaphylactic symptoms largely or entirely

In addition a partial or complete loss of the capacity of a tissue to react under another different antigen antibody stimulus may exist for a time following a reaction a fact strongly suggestive of the exhaustion of some tis ue component required for the reaction (cf Nicoll and Campbell 1940 Chase 1947) Other treatments that result in a quite temporary nonspecific desensitization of the guinea pig are described by Hill and Martin 1932 and by Kellaway and Cowell 1922 (cf Moldovan and Maier 1941)

Desensitization The introduction of very small amounts of antigen beneath the skin given as a series of injections of increasing amounts over the course of a few hours without the production of symptoms serves to desensitize an animal and to render it immune to shock for some days (Besredka) In desensitization (cf Longcope 1923) it is commonly said that the available antibody is neutralized but some alteration in the reactivity of the tissue component may possibly be involved as well Practical use is still made of this procedure when sensitized animals must receive further injection (Kay 1940) it has been applied successfully to man also but it is hazardous (Friedberger and Mita 1912 Mackenzie 1921 Ratner 1943) Eventually however with cessation of injections the anaphylactic state becomes reestablished *there is no known technic for effecting a permanent desensitization*

Inhibition and Shock by Haptens With the advent of artificial conjugated antigens owing to the work of Landsteiner (see p 154) it became possible to examine by anaphylaxis the serologic specificity lent to proteins by the attachment of certain types of chemical radicals which have come to be called haptens It turned out that following sensitizing injections with such a grouping combined with one protein fatal shock could result from subsequent testing with the same grouping combined with an entirely different protein When now the hapten itself or a simple derivative of it was injected intravenously prior to the shocking conjugate a state of specific inhibition of shock was to be found akin to inhibition in vitro of a precipitating antigen antibody system that is the small molecule could compete successfully with the large protein complex for the antibody and so delay the requisite interaction between antibody and the full antigen In some cases the effect appears to be a simple inhibition with the tissue retaining its full reactivity in others there is evidence for specific desensitization as well

On the other hand there are special instances in which the haptenic structure is itself capable of inducing shock in sensitized animals Thus microbial polysaccharide haptens (from *Aerobacter aerogenes* Friedlander s bacillus and a yeast) were shown by Tomcsik and Kurotchkin (1928) to shock guinea pigs passively sensitized with the corresponding antibacterial sera and Avery and Tillett (1929) secured similar effects with type specific polysaccharides of pneumococci including the nitrogen free Type 3 polysaccharide Similarly azodyes in particular tho e containing two haptenic groupings to the molecule (made by such devices as a double coupling to resorcinol) and having probably a larger structure owing to aggregation of molecules of the *dis* azodye can cause anaphylaxis

transferred within a given species, but also in certain instances with heterologous antibody as well[3]

By far the most productive have been the findings of Kabat and his co workers (dating from 1942) who have employed quantitative methods of immunochemistry in resolving long debated questions

The amount of guinea pig antibody that is required to lead to fatal anaphylactic shock has been measured quantitatively by Kabat and Boldt (1944) for an ovalbumin anti ovalbumin system and is extremely small—5 to 30 μg antibody Nitrogen or less than 0 2 mg antibody globulin With especially prepared guinea pig antisera amounts as little as 0 2 ml may contain this amount of anti body but with the serum of guinea pigs as ordinarily sensitized 2 to 4 ml are required for full sensitization It will be apparent that only a fraction of the administered antibody can become associated with for example, a uterine horn that is removed for the purpose of organ anaphylaxis Grabar 1953 has esti mated the amount as 0 01 μg antibody Nitrogen

Guinea pig tissues can be sensitized like wise by antibody produced in the rabbit Since rabbits yield antisera of high titer rabbit im mune sera are employed usually for passive anaphylactic sensitization The minimal amount of rabbit antibody necessary to induce fatal anaphylaxis has been measured quanti tatively by Kabat and Landow (1942) and is of the same order of magnitude (about 0 03 mg antibody Nitrogen) as is required with guinea pig antibody however this may be contained in as little as 0 02 ml rabbit immune serum Rabbit antibody is equally effective whether used as the common pre cipitating variety or as nonprecipitating or 'univalent antibody (see p 129) (Kabat and Benacerraf 1949)

Isolated normal tissue (uterine horn) may be prepared to undergo anaphylactic responses by being bathed in antisera (Dale 1913 Hartley 1939 Kulka 1943)

In the guinea pig the anaphylactic state can be inherited from the mother apparently an instance of passive transfer of antibodies as the sensitivity is gradually lost during the first 6 to 10 weeks of life

The guinea pig is essentially insusceptible to sensitization by antibody produced in the horse, cattle, the chicken or the rat Some human sera can establish passive anaphylac tic sensitization — human antitoxic (diph theric) sera (Neill Sugg and Richardson 1932 Kuhns and Pappenheimer 1952) specimens of serum sickness sera (Longcope and Rackemann, 1918 Tuft and Ramsdell 1929), and certain specimens from asthmatic cases (Ramsdell, 1930), but human sera con taining solely reagins do not do so

For establishing the anaphylactic state pas sively it is advisable to allow a latent period of some hours (2 to 18) between the giving of antibody and the subsequent challenge of the guinea pig, although there may be heredi tary differences in strains of guinea pigs (Zin sser and Enders 1936) No latent period need be observed, however, if sufficiently large amounts of immune serum are employed or if one injects soluble preformed antigen anti body complexes prepared in antigen excess (Germuth and McKinnon, 1957) In rein vestigating the relation between the latent period and the amount of antibody (rabbit antiovalbumin), Benacerraf and Kabat (1949) using quantitative methods and con stant antigen found that at least 40 fold the amount of antibody was needed to result in immediate shock than was required when a 5 hour latent period was allowed

From the start the latent period was in terpreted as being the time required for anti body to become fixed to tissue cells Indeed the process involves more than a single step (von Fenyvessy and Freund 1914) for the amount of antibody necessary to determine the anaphylactic state can be taken up from the blood stream within an hour long be fore the sensitivity itself may be demonstrable Benacerraf and Kabat (1949) reiterate that the reaction evidently taking place may well be the fixation of antibody to cells since the sensitizing amount of antibody becomes pro gressively less as the latent period is extended and the minimal shocking dose of antigen decreases at the same time

The sessile state of the anaphylactic anti body was considered beyond question when it was found that the perfused uterus of an ac tively or passively sensitized guinea pig can react in the Schultz Dale experiment (Dale 1913) However this argument was weakened

3 A most useful extension of passive transfer is the technic of inducing localized cutaneous reactions that has come to be called *Passive Cutaneous Anaphylaxis* (p 163)

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directly, without use of protein carriers, this matter has been pursued by Campbell and McCasland (1944), who showed further the inhibiting effect of univalent haptens

Reversed Anaphylaxis The production of the syndrome of anaphylaxis in a normal animal by injecting first the antigen and then the corresponding antibody has been termed 'passive reversed anaphylaxis' It has been attained in the rabbit, the mouse and even the guinea pig, but commonly requires the use of very large amounts of antibody A special instance, described as reversed anaphylaxis is presented by those animal species in whose tissues Forssman heterophil antigens are present for instance the guinea pig If an antiserum produced against some other heterophil antigen is introduced intravenously (such as an antiserum against sheep erythrocytes developed in the Forssman negative rabbit) the antibody reacts with the guinea pig s tissues and leads to death with pronounced anaphylactic features however the isolated guinea pig uterus is said not to respond to such antisera

Anaphylaxis in the Dog

Some of the most profound observations in anaphylaxis have been made on the dog In this species several injections of native protein are usually required to sensitize adequately

When shock is produced in the dog as Manwaring showed the liver plays a chief role the liver of the sensitized dog brought into shock while it was joined to the circulation of a normal dog produced a soluble agent that caused even the normal tissues to respond The active material was proved to be histamine (Dragstedt et al Code [Code 1939, 1944]) and has been considered to be adequate in amount to account for the vascular reactions

Injection of antigen by the intravenous route is followed by restlessness then vomiting salivation and diarrhea (at time bloody) and finally profound collapse with loss of muscle tone and slow, often labored respiration at the same time the blood pressure and the body temperature decrease markedly As with the guinea pig the released heparin causes a loss of coagulability of the blood and there is a decrease in serum complement a pronounced leukopenia in the peripheral blood reflects the elective retention of polymorphonuclear leukocytes in the lung capillaries where masses of white cells adhere tenaciously (Dean and Webb 1944)

In the most acute instances of anaphylactic death necropsy discloses chiefly an enormously distended and congested liver (indeed, it may contain as much as 60 per cent of the blood) there is, in addition, a general congestion of the gastro intestinal tract when shock has lasted for several hours

During shock, reactions have been shown in various organs in situ (Manwaring et al 1925), and the resemblance to guinea pig anaphylaxis will be apparent there is contraction of uterus, intestinal tract (chiefly the colon and the rectum) and urinary bladder and some degree of bronchoconstriction has been reported as well The isolated liver perfused with antigen in whole blood is found to release histamine and heparin readily (Rocha e Silva) but not with Locke s solution (Scroggie and Jaques 1949) in all probability because the physiologic state of the tissue component is not maintained adequately

The anaphylactic state may be induced passively by administration of antiserum, and it is stated that reactions can be secured without any latent period (Sherwood et al 1946)

Anaphylaxis in the Rabbit

Several preparatory injections of antigen are usually required to sensitize rabbits and even then systemic shock is not experienced regularly Consequently less is known about the conditions for producing systemic shock in the rabbit than in either guinea pig or dog It is certain that the concentration of circulating antibody must be high The cause of death was shown by Coca in 1919 to be a consequence of obstruction of the pulmonary arterioles, causing right sided dilatation of the heart with death attributable to secondary heart failure While Coca pointed to contraction of the abundant smooth muscle around the pulmonary arterioles as the source of their obstruction McKinnon et al (1957) have recently emphasized the occurrence of obstructing intravascular amorphous thrombi in the pulmonary circulation that as shown by the use of fluorescing antigen are in actuality antigen antibody precipitates

The sequence of events upon intravenous injection of antigen differs from both those of the guinea pig and the dog there is arrhythmic respiration, then panting with tem

porary hyperemia in the ear followed by arteriolar contraction and blanching the rabbit becomes weak and collapse gives a few convulsive movements defecates and dies suddenly with head retracted and eyes in exophthalmos Bronchospasm is absent but the lung is nonetheless the main organ of shock

At necropsy there is found extreme dilatation of the right half of the heart and engorgement of the inferior vena cava the portal vein and the liver Owing to obstruction of the pulmonary arterioles the blood pressure in the carotid falls greatly while the pulmonary pressure increases markedly Coagulation of the blood is somewhat delayed The stickiness of leukocytes and the clumping together and clinging of these cells to the vascular endothelium which has been mentioned as occurring in the dog lung may be observed in newly regenerated blood vessels of the rabbit ear when antigen enters the circulation (Abell and Schenk 1938) and has been shown to take place in the sensitized rabbit lung under perfusion with antigen and whole blood The clumps of leukocytes are even sufficient to obstruct small vessels

While anaphylaxis in isolated and perfused organs is not well demonstrable constriction of isolated strips of the pulmonary artery has been shown (Grove 1932) and anaphylactic constrictions have been shown in the vessels of the perfused rabbit ear (Abell and Schenk 1938) in the lung there is a resistance to the flow of saline perfusion fluid containing antigen (Friedberger and Seidenberg 1927)

As with other animals temporary anaphylactic desensitization seems to be attained by a few subcutaneous injections of small amounts of antigen in the course of 3 or 4 hours this procedure is employed at times in resuming intravenous injections with certain antigens following periods of rest in the well known alternation of courses of injections and resting periods so commonly practiced with rabbits for the purpose of securing potent antisera

In the actively sensitized rabbit there is a significant parallelism between the severity of shock and the concentration of circulating antibody (Jackson 1935) although discrepancies are sufficient to indicate the participation of other factors than the amount of circulating antibody in determining the outcome of anaphylactic shock (cf Kabat 1947) The reaction of antigen and antibody in the blood stream may well be dominant over the union of antigen with antibody in intimate relation with the tissues Indeed passive sensitization is possible with adequately large volumes of serum (Arthus 1919) and may be demonstrated without a preliminary incubation period Further certain of the symptoms of nonfatal shock are produced in normal rabbits upon the injection of antigen antibody precipitates (McKinnon et al 1957) Platelets of the rabbit are rich in histamine and serotonin (Humphrey and Jaques 1954) and release physiologically significant amounts of histamine into the plasma under the stimulus of antigen antibody interaction not only upon addition of antigen to fluid blood or to the buffy coat thereof taken from a sensitized rabbit (Katz) but even upon simultaneous addition of antigen and antibody to normal rabbit blood (Dragstedt) and serotonin likewise (Humphrey and Jaques 1955) It is interesting to note that the histamine liberator 48/80 does not release histamine from rabbit platelets

In systemic shock also histamine is liberated into the plasma but this circumstance was not appreciated for long since white cells and platelets leave the circulation during shock and determinations of histamine levels on whole blood showed a histamine deficit during anaphylaxis As Feldberg suggests the main release of histamine may occur in juxtaposition to the pulmonary arteries

Anaphylaxis in Other Animals

Anaphylactic shock in other animals will be mentioned only briefly While virtually all species exhibit the phenomenon in many there is no dominating shock organ A rough correlation has been pointed out between the ease of establishment of the anaphylactic state and natural sensitivity to histamine (Table 14) Thus the rat and the mouse highly resistant to histamine intoxication undergo systemic anaphylactic shock far less readily than the guinea pig But as indicated before the balance of factors can be quite complex

The rat does not become sensitized readily unless special methods are used (Lipton Stone and Freund 1956) Both the leukocytes and the mast cells of the rat are rich in histamine to which the rat is essentially resistant it has been suspected that anaphylaxis in this species is related to liberation of serotonin, which is

TABLE 14

	GUINEA PIG	DOG	RABBIT	MOUSE	RAT	MONKEY	HORSE	PIGEON
Evidence of antibody fixation to tissues[1]	Uterus gut lung vessels gall bladder heart coronary arteries urinary bladder		Strips of pulmonary artery blood vessels of lung and ear (antibody retention by skin and uterus)					
Antibody in excess	Inhibitory		Necessary	Necessary				
Duration of sensitivity[2]	Many months			Brief	Brief			
Incubation period in passive transfer	Several hours	None	None	None				
Onset of shock (active or passive)	3 to 6 min	10 sec (acute)	15 sec	12 to 60 min		30 min to 24 hrs	8 min	
Situs of active shock	Lung broncho constriction	Liver	Pulmonary arteries mechanical heart failure Liver				? (Dyspnea)	
Situs of protracted shock	Lung edema Congestion of viscera Liver (?)	Congestion of viscera						

[1] Reaction to Ag in saline after perfusion [2] Established without use of adjuvants

TABLE 14 (*Continued*)

	GUINEA PIG	DOG	RABBIT	MOUSE	RAT	MONKEY	HORSE	PIGEON
Sensitivity to histamine[3] (mg/kg body weight)	0 3 0 4	3	0 6 3 0	250 300	1/0 500	50		1 5
Sources of histamine	Aorta lungs uterus seminal vesicles (liberation by perfusion *with antigen* in saline)	Liver (liberation by perfusion with antigen in whole blood)	Platelets and/or leukocytes (vessels)?	(Few)				
Role of histamine	Highly probable role not exclusive	Certain	Highly probable	Very uncertain	Very uncertain			
Role of serotonin	Possible		Probable					
Sources of serotonin	Mast cells?		Platelets					
Sources of choline acetylcholine	Heart							
Physiological acutance					Adrenalectomy use of *H pertussis* vaccine			

[3] Data compiled by R L Mayer

also present in high concentration in the mast cells

Mice can be sensitized by several injections of fluid antigen (cf Cameron, 1956) but much more easily by alum precipitated antigen (Solotorovsky and Winsten 1953) Passive sensitization is possible with potent antisera of rabbit and guinea pig and no incubation period is necessary (Burdon, 1946) McMaster (cf McMaster and Kruse, 1951) has employed the ear of the sensitized mouse for direct observation of vessel spasms during anaphylaxis, arteriolar and venous spasm following intravenous injection of antigen is dramatic, the capillaries and the lymphatics remaining unaffected Several procedures enhance the anaphylactic sensitivity of the mouse adrenalectomy shortly before sensitization or pretreatment with *H pertussis* vaccine (Kind and Parfentjev 1951 review by Pittman, 1957) The latter procedure renders mice more than 10 fold as sensitive to histamine as they are normally (Table 14) Since the amount of histamine that exists in the mouse is thought to be only one tenth of the quantity needed for fatal histamine shock a role for serotonin or other active materials has been postulated in this species also

Anaphylaxis in the monkey has been studied by Kopeloff and Kopeloff (1939) and by Kinsell Kopeloff et al (1941) Sensitization is not easily accomplished With horse serum as antigen the time of reaction to the shocking injection varied from 30 minutes to 24 hours edema and hemorrhage of the lungs and the intestinal tract and of the skin were observed when death did not occur early

Anaphylaxis in the horse and the calf has been studied by Code and Hester (1939) with sensitizing and shocking procedures patterned on those commonly used with small laboratory animals Progressive dyspnea increased peristalsis and sweating were seen in 2 horses and respiratory difficulty appeared to be the cause of death in 1 horse which died in 8 minutes In horses studied by Gerlach (1922) and Ritzenthaler (1924), urticarial responses were prominent and there was marked edema of the legs It may be mentioned that horses exhibit dyspnea very commonly during injections with bacterial vaccines (pneumococci) and may show prompt accumulation of fluid in the lungs

Birds may be sensitized and in particular several studies have been carried out in the pigeon the anaphylactic contractability of the isolated circular muscle of the pigeon s crop has been suggested as a laboratory tool

Anaphylaxis in Man

Systemic reactions apparently identical with instances of anaphylactic shock of the lower animals have been encountered in man While most instances—and the gravest—have occurred in persons exhibiting so called ' clinical allergies of the immediate type and possessing circulating antibodies of the special reaginic' type (see p 169), ostensibly anaphylactic symptoms have resulted from spaced intrathecal injections of antimeningococcal horse serum employed for therapy in meningitis ' Reversed anaphylaxis" has been implicated in uterine contractions that have followed the administration of specific horse antipneumococcal sera to women sick with lobar pneumonia and consequently possessing pneumococcal polysaccharide

Individuals who have become sensitized to foreign serum because of prior administration and—ostensibly because they belong in the ' atopic category—give the ' immediate type of reaction upon skin testing are most prone to systemic accidents The most severe response comprises collapse, fall of blood pressure tachycardia dyspnea of the asthmatic type suffusion of the face urticaria with giant wheals, and sometimes marked edema of the entire body (Longcope and Winkenwerder 1941) in fatal cases death may occur within a few minutes or as late as 24 hours In short it has been stated that anaphylaxis in the human being may resemble any animal type or a combination of them The literature has been reviewed by Ratner (1943)

Bronchiolar constriction is not invariable and there is no one dominant shock organ Most of the fatal and grave incidents occur upon administration of horse serum to people having a pre existing clinical sensitivity to horse dander that is ' horse asthmatics (Ratner and Gruehl 1929) Here postmortem examination is apt to reveal distended lungs splanchnic dilatation and at times engorgement of the liver The hypertrophy of bronchial musculature frequently developing in such subjects has been interpreted as rendering them particularly liable to respiratory symptoms and has been likened to the condition normally existing in guinea pig lung The tissue changes the presence of passive transfer antibodies and the type of response are like those occurring in pollen hay fever where similar accidents are experienced upon em

ploying an overdose of pollen extract Kallos and Kallos Deffner (1937) found evidence of enlarged and emphysematous lungs in autopsy records of patients dying in a thmatic attacks and they pointed out that the special histologic character of the alterations in *Asthma bronchiale* rests on a peculiar allergic reaction of the bronchial wall and of the lung tissue *(translation) Concomitant changes in lym* phoid tissue and adrenals are also under eval uation (Winer et al 1950)

Anaphylaxis has been noted following repeated injections of tetanus toxoid and of a prophylactic preparation of alum precipitated diphtheria toxoid and pertussis antigen (Werne and Garrow 1946) in the latter *instance the role played by hereditary fac* tors seems to be obvious With this prepara tion identical twins died in delayed shock 16 and 24 hours re pectively after the second in jection Both children showed histopathologic evidence of acute vascular injury and edema constricted arteries and tissue eosinophilia

Other odd and unpredictable but compre *hensible serum accidents may occur* (Fawcett and Ryle 1923)

LOCALIZED TISSUE DAMAGE

When small amounts of antigen or allergen are introduced into the tissues of a sensitive individual contact is localized the rate of absorption or dissemination is retarded by tissue barriers and by existing antibody mechanisms and systemic reactions need not be excited A local reaction is initiated and kept stimu lated for a time Histologically the allergic inflammation starts almost at once In pecial cases when time is required for some altera tion of the administered allergen by interme diate metabolism or coupling with body substances there is an artificial time lag Certain of these reactions are frankly evanescent others (the so-called Arthus type) require a day or so for full immunologic development The difference between these varieties of ex pression probably depends on availability of *excess circulating antibody ver us a special* type of skin sensitivity perhaps the princi ples are those that appear in Passive Cutane ous Anaphylaxis

Passive Cutaneous Anaphylaxis

A novel type of evanescent kin reaction appearing to involve an intimate antibody tissue relationship was de cribed by Ramsdell (1928) and elaborated by Cha e (1943 1947) and Ovary (1951 ee Ovary and Bier 1953) When suitable dilute antibody is injected into the skin of guinea pig or rat a latent period allowed for fixation of antibody and antigen introduced systemically there occurs an imme diate and sharply delimited reaction reaching maximal erythema and edema within 20 min utes and then fading away All the e features are constant with an anaphylactic type of reaction occurring within the skin instead of systemically the term *Passi e Cutaneous Anaphylaxis* or PCA was introduced by Ovary (Owing to the capillary damage that occurs during the reaction dyestuffs of high molecular weight can pass from the blood stream into the affected tis ue and antigen i often mixed with Evans Blue prior to intra venous injection) The guinea pig accepts anti body produced in the guinea pig the rabbit or the human being rabbit antibody is known to be accepted by the rat the mouse and the hamster

While this technic has been developed chiefly for delicate measurements of antibody (0 003 μg or less of antibody nitrogen in jected into the skin in 0 1 ml volume causes local sensitization) only facets that are re lated to allergy are of concern here (1) Com *plement appears to play a role in the reaction* (Osler et al 1957) (2) Following a reaction there occurs a local exhaustion within the area of skin of some component that may not be replaced for as long as 7 days (Chase 1947) (3) Excess antibody introduced into the skin can establish systemic anaphylactic sensitiv ity and an onset of systemic shock during the test with antigen will *negate development of* the local reaction (4) Some reactivity remains in the prepared area of skin as long as 5 to 10 days when homologous antibody is used although the sensitivity maximal within 6 to 17 hours after injection decreases with time (5) The reactions are usually to be induced far more sharply in the skin of a recipient guinea pig than in the skin of the antibody donor probably because antibody present in the latter s circulation blunts the anaphylactic type reactivity by overlaying an Arthus com ponent

The Arthus Reaction

A severe type of damage resulting from the *injection of antigen into the tissues was found* by Arthus in 1903 Rabbits repeatedly in

jected beneath the skin with horse serum came to respond with progressively more intense reactions at the site of each succeeding injection The initial response was a transient local swelling, the ultimate response was hyperemia and edema of the skin, followed by hemorrhage and intense induration requiring a day or more to develop and progressing to deep necrosis and sloughing The intensity of the reaction in actively sensitized rabbits was found to vary with the antibody level in the serum when antigen was injected intracutaneously (Culbertson 1935) the occurrence of deep necrosis and slough requiring 0 75 to 1 0 mg antiovalbumin protein per ml serum

Not only the skin but also many other sites develop an inflammatory response when antigen is injected locally into rabbits—stomach submucosa kidney liver, brain lung testicle, joints ligatured blood vessels (Seegal, 1935 Opie 1936 Cannon Walsh and Marshall 1941)—and striking inflammation of the pericardium and the myocardium has followed instillations of antigen into the pericardial cavity (Seegal 1935)

The Arthus reaction actually is initiated promptly but several hours can be needed for development of a macroscopically visible reaction The eventual necrosis and sloughing is a direct consequence of the initial vascular damage and thrombosis

Microscopically even within an hour after subcutaneous injection of antigen (Gerlach 1923) the site of deposition may exhibit evidence of marked alterations in capillary permeability—intense swelling of connective tissue fibrils with compression of the blood vessels a surrounding massive edema and immobilization of leukocytes The process is at maximal histologic expression at 24 hours when there is necrosis of the arteriolar vessel walls and consequent hemorrhage fibrinoid degeneration in the connective tissue and adventitial inflammation and edema

These earlier stages have been studied anew by Stetson (1951b) with interesting findings his animals probably possessed a somewhat lesser degree of sensitization than Gerlach s Following injection of *antigen into the skin* there occurs within 15 minutes a pronounced but transient leukopenia the cells and the platelets being sequestered by adhesion to the endothelium of small vessels aggregates of leukocytes and platelets (which form even in vitro when antigen is added to whole blood from the sensitized rabbit) appear at the site of injection and form actual thrombi assisted perhaps by a fine layer of fibrin along the walls of the vessels (cf Nolf, 1910) With the continued increase in numbers of leukocytes over *a period* of several hours there is an abnormal accumulation of lactic acid owing to aerobic glycolysis by the "exudate leukocytes As Stetson has emphasized the parallelism of the Arthus reaction and the Sanarelli Shwartzman phenomenon in this sequence of events leading to endothelial damage becomes obvious In view of the early leukopenia the novel suggestion was made that the local Arthus effect may require a primary reaction between antibody in the blood stream and antigen escaping from the skin depot

The acute phase appears to be over by 8 *hours (Gell and Hinde, 1954)* many polymorphonuclear leukocytes then being degenerate at this time, a circumferential mononuclear cell reaction is rather conspicuous *In the zone of the latter cells immature plasma* cells and eosinophils are seen by the fourth day, and thereafter progression to plasma cells proceeds apace While several aspects of this mononuclear contribution are seen in tuberculin reactions the final appearance of plasma cells is confined to the Arthus reactions

Therefore the physiologic changes that were noted previously in rabbits undergoing systemic shock are associated with the development of skin lesions local aggregates of leukocytes and platelets the meeting of antigen and antibody under conditions in which histamine and possibly serotonin are liberated from circulating platelets and cells vasodilatation, injury to the vascular endothelium, and *edema It may be noted* that the administration of heparin prior to injection of antigen is able to mitigate the intensity of Arthus reactions in cutaneous tissues (Gregoire 1946) and in the eye (Bick and Wood, 1950), perhaps because of its affinity for prothrombin and "complement

The cells of the tissues apparently are not intrinsically sensitized but derive their capacity to react from antibody in the environment (cf Opie 1924 Benacerraf and Kabat 1950) Cells of the sensitized *rabbit survive normally* in tissue culture in contact with the specific antigen, and there is lack of injury when antigen is injected into the relatively avascular cornea (Aronson 1933, Rich and Follis 1940)

The capacity to react as in anaphylactic transfer may be transferred to normal rabbits by injection of the serum from actively sensitized rabbits (Nicolle Opie 1924a). If such serum is given in quantity intraperitoneally or intravenously a reaction may be elicited anywhere on the entire skin area of the recipient rabbit whereas if the antibody is injected into the skin a local reaction will occur at that site *if very shortly thereafter antigen is given* either locally or intravenously.

The amount of antibody required for transferred Arthus reactions in the rabbit has been measured by Fischel and Kabat (Kabat 1947) and is found to be 1 4 mg antibody protein for maximal and 0 15 mg for minimal reactions when antibody is deposited intracutaneously antigen having been injected intravenously 30 minutes before (cf Culbertson 1935 Cannon and Marshall 1941). Such transferred Arthus reactions performed on the skin of the back develop slowly but it is interesting to note that the same procedure carried out on the rabbit's ear—a tissue in which direct Arthus reactions are not encountered (Gerlach 1923)—results in immediate type evanescent responses (Ramsdell 1928).

The amount of antibody required for transferred Arthus reactions in the guinea pig has been measured likewise (Benacerraf and Kabat 1950) and is fairly close to the values found to hold for the rabbit—1 2 mg antibody protein for intense reactions and about 0 06 mg for minimal reactions rabbit and guinea pig antibody (antiovalbumin) are found to be quantitatively equivalent but rabbit nonprecipitating or univalent antibody is markedly inferior.

In a comprehensive study Benacerraf and Kabat (1950) further demonstrated that no latent or preparatory period was required before *maximal reactions could be obtained* that antibody deposited in normal skin largely filters from it freely and soon becomes distributed generally and that the attachment of circulating antibody to intracutaneously injected antigen is nearly maximal within the first 15 minutes.

Among the laboratory animals the Arthus phenomenon comes to fullest expression in the rabbit but it occurs usually after many preparatory injections in other species including horse (Gerlach 1922) monkey (Kopeloff and Kopeloff 1939) guinea pig (Arthus Cannon and Marshall 1941) and man it cannot be induced readily in the rat and the mouse except in the lip owing to the number of mast cells found there (Freund and Stone 1956). The well developed reactions of the rabbit are referrable to the high antibody titers encountered in this species and may be related also to the content of available physiologically active material liberated by white cells and platelets in the presence of antigen and antibody (Katz Dragstedt Humphrey and Jaques (1954 1955). A gradual onset of *Arthus-type sensitivity occurs in the guinea* pig (a poor producer of antibody) as the number of antigenic stimuli increase the earliest representing possibly a sort of active cutaneous anaphylaxis later ones exhibiting a soft edema that persists for a few hours and the ultimate reactions showing both a broad edema and centrally petechial hemorrhages that rapidly become confluent within the first few hours and lead terminally to deep necrosis and sloughing (Kellett 1930).

Description of the Arthus reaction as local anaphylaxis is misleading and should be avoided.

Anaphylactic and Arthus type sensitivities can be differentiated clearly in the guinea pig owing chiefly to the precise studies of Kabat and his co-workers with carefully measured antibody. The distinguishing features are these (1) There is no need of a latent or incubation period before demonstrating passive Arthus reactivity whereas there can be such a requirement for passive anaphylactic sensitization with the lapse of some hours both states coexist (2) The amount of antibody required upon intravenous injection for Arthus type reactivity to be established is considerably greater than for fatal anaphylactic sensitization (3) Nonprecipitating or univalent rabbit antibody leads only to a low grade Arthus reactivity although it is as effective as precipitating antibody in transferring anaphylactic sensitization (4) Obversely Arthus-type sensitivity of moderate intensity is established by injecting antibody of the horse (one reacting with pneumococcal carbohydrate) which is not able to induce anaphylactic sensitivity.

Lesions of the Arthus type have been reported in man usually upon injection of foreign serum and in instances in which a prior administration has given some urticarial signs indicative of an existing sensitivity (Kojis 1942 Ratner 1943).

Local reactions of this sort have been re

produced unintentionally in children by giving them subcutaneous injections of antitoxic horse serum at 3 week intervals (Lucas and Gay 1909) Between the second and the sixth injections there appeared, in addition to scattered cases of generalized urticaria the characteristic local changes—edema tenderness erythema and marked, persistent induration or eventual necrosis At times when therapeutic injections of immune horse serum have been given to severely ill, sensitive patients the reactions have been extraordinarily extensive and sometimes have even showed black dry eschars over large areas [Gatewood and Baldridge (1927) Trumpeer et al (1931)] Extensive slough of the skin and the subcutaneous tissue has been noted (Kohn McCabe and Brem 1938) Whether or not there is some auxiliary role attributable to concomitant bacterial disease is unknown for example participation of the Sanarelli Shwartzman phenomenon (p 190) *Passive transfer of local* serum reactivity in man has been reported by Kojis (1942)

Immediate Type Responses of Man

From the studies on animals, it is clear that the occurrence of the early type of reaction coincides with a presence of antibodies that *are capable of sensitizing normal individuals*—according to the route of injection—either to react anywhere on the skin or in a local area the chief exception is a persisting sensitivity in *some animals principally the guinea pig for* many months after the antibody has become undetectable in the serum and passive transfer *is no longer possible With discovery in 1921 of the human 'reaginic antibody in individ*uals possessing a cutaneous reactivity of the so called wheal and flare type and in demonstration by Prausnitz and Kuestner of pas*sive cutaneous transfer* (see p 168) an important link was closed

In thus linking together all instances of transfer of sensitiveness by serum and in seeing therein a general principle of immunology we must make it clear at the outset that questions regarding the nature of the antibodies concerned, and which subjects are capable of producing them are not to be resolved simply Also it must be reiterated that some forms of hypersensitiveness in man particularly to foods—properly to be regarded as reactions of early type—can occur without evidence of circulating antibody and without positive cutaneous reactivity to the offending allergen ("familial nonreaginic food allergy' of Coca) And one must understand that there are wheal reacting allergies in which circulating antibodies are not demonstrable by current technics

Studies on human beings reveal a capacity to form several sorts of antibodies—precipitins and agglutinins of several types, co precipitating antibody, the so called ' blocking' antibody (p 171) and reagins Reagins are usually encountered in association with 'spontaneous allergic states such as seasonal coryza and asthma and some kinds of eczema but are hardly to be found in urticaria and angio*neurotic edema Curiously only the reagin,* among human antibodies injected into normal skin, remains detectable in a local site it is still more curious that apart from anti*bodies formed in serum sickness there is corre*lation only between existence of clinical disease and production of the reaginic type of antibody

*Reagins, related to inhalant and food aller*gens especially were found by Grow and Herman (1936) when no corresponding clinical disease was apparent (the latter being *judged solely by natural and not experimental* conditions of exposure) Although the possession of reagins seemingly would suggest a potentiality for a later clinical disorder, the *presence of a special reactive site* (shock tissue) has been said to determine the event of disease not the mere presence of reagins in *the circulation However the high correlation* between reagins and clinical disease in wheal reacting allergies does suggest that one cannot attribute the decided influence of heredity [Cooke and Vander Veer (1916) Spain and Cooke (1924)] solely to tissue factors

The inheritable property is a susceptibility for sensitization only, since the allergens concerned and the type of clinical manifestations vary among sibs and between children and their parents In some cases previous contacts cannot be traced especially in young babies it is possible that sensitization can occur through traces of substances provided by the maternal diet The opportunities for undisclosed sensitization naturally increase with age

To connote sites of predilection for allergic reactions seen in various individuals allergists have introduced the term shock organ —the bronchial mucous membrane (asthma) the mucous membrane of the eyes and the upper respiratory tract (hay fever, allergic coryza or rhinitis) the endothelium of the superficial vessels in the upper cutis (urticaria wheal reacting allergies) the gastro intestinal tract (forms of food allergy) and the like The im plication that antibodies are distributed un equally among the various tissues suggests a need for critical experimentation That the tissue component can become altered patho- logically through undergoing repeated allergic responses and can become extraordinarily hyperreactive to physiologically active sub- stances liberated in the course of antigen antibody tissue reactions is clearly to be seen in the lungs of asthmatic individuals To some extent the concept of a particular shock organ will be an artificiality for the observed reaction may reflect the usual route of contact with the allergen vis a vis the intensity of the sensitivity rather than solely the existence of loci with exaggerated sensitivity For example a person who suffers only from hay fever under natural conditions of exposure will sometimes exhibit instead an attack of asthma or a generalized urticaria when his allergen is injected for prophylactic purposes

Serum Disease and Its Analogues After the discovery of diphtheria antitoxin the illness known as serum sickness was observed in patients treated by therapeutic injections of antitoxic horse serum with the employment of horse antistreptococcal antimeningococcal and antipneumococcal sera in still larger doses the case incidence increased markedly [4] Within 7 to 12 days—closely similar to the time re quired for development of the anaphylactic state in the guinea pig—there was a general ized swelling of lymph nodes urticarial or erythematous eruption with itching and often edema of the eyelids the face and the ankles in severe cases arthralgia and fever followed the eruption

The disease appears as a consequence of the interaction between specific antibodies just formed and the remaining traces of foreign proteins The disease may be transient (the usual period is slightly over 2 days) or it may last up to 2 weeks With use of crude horse serum there may be one or more recurrences of the eruption attributable to the fact that antibody formation to the various serum pro- teins is established at different times just as occurs in serum sickness following injection of isolated beef gamma globulin (7 days) and beef albumin (14 days) [Hawn and Janeway 1947]

If the antibody producing mechanism has been established by some previous but not too recent injection a new administration of horse serum will bring on the same train of events after a shorter interval 3 to 5 days (accel erated reaction) paralleling the recall phe nomenon observed when animals are restimu lated with antigen after a rest period

The recognition of serum sickness as an allergic disease was accompanied by the dis covery in patients sera of antibodies giving precipitation in vitro with horse serum and anaphylactic sensitization in the guinea pig (Longcope and Rackemann 1918 Tuft and Ramsdell 1929) and like findings appeared in certain allergies to common foods (Schloss 1912) These antibodies being precisely those that were to be expected in view of the anti genic stimulus the disease came to be regarded as an anaphylactic or Arthus type allergy (cf Table 13) At the same time skin sensitizing antibodies having the character istics of reagins are often present (de Besche 1923) and likewise pointing to reagins is the fact that skin sensitivity persists long after classic antibodies can be found in the serum Evidently both skin sensitizing reagins and classic antibodies occur together In this re gard it may be mentioned that the antibodies present in serum sickness can be demonstrated advantageously by reversing the sequence of injections used in the Prausnitz Kuestner technic probably because antibodies other than the special skin sensitizing antibody can participate (Voss 1938 Wright and Hopkins 1941 Karelitz and Glorig 1943)

The incidence of serum sickness today is reduced because of a lessened use of sera of animal origin and because of chemical modi fications to which some immune globulins can be submitted (Parventjev process) The in creasing frequency of injection of viral mate rials prepared in the embryonated egg and in rodent brain especially in conjunction with adjuvant procedures designed to *enhance*

[4] The monograph by von Pirquet and Schick (1905) is comprehensive see Longcope and Winkenwerder (1941) and Ratner (1943)

antigenicity, could lead to a new wave of sensitization to foreign proteins In practice the avoidance of adjuvants and the taking of careful case histories prior to injection have permitted suitable immunization of troops with several sorts of viral materials

Reinjection at a time when the sensitivity is sufficiently high may cause local immediate reactions within 15 minutes to an hour, and also general immediate reactions occurring within some minutes to 12 hours generalized immediate reactions are often severe and occasionally may prove to be fatal but are said *to occur chiefly in persons that are known* to be sensitive to horse dander ('horse asthmatics')

Testing for immediate type sensitivity is generally practiced by cautious intracutaneous injection and conjunctival instillation of dilute material prior to therapeutic administration of antisera of animal origin However as Longcope emphasizes (1943) there should be employed in addition a preliminary intravenous *test with 0 1 ml or less of the material* untoward reactions may occur in the absence of positive cutaneous and conjunctival tests as is comprehensible in view of the several varieties of antibody mentioned below

In recent years clinical manifestations closely resembling "serum disease have been seen as a consequence of drug therapy for instance with the sulfonamides the arsphenamines penicillin and others (Longcope 1943 cf Carr, 1954) A formation of derivative antigens in vivo between tissue component and chemical probably explains the sensitizing effect and could account for the *frequent failure to find antibody or to reveal* skin sensitivity by use of the chemicals themselves The pattern of 'serum disease is manifest in the occurrence of fever skin eruptions (erythema urticaria or purpura) and lymphadenopathy and as an aftermath of sensitization both accelerated and immediate types *of response are* to be encountered upon readministration of the drug

Wheal and Flare Reactions The early type of cutaneous response to intradermal injection of allergenic excitant is far more pronounced in man than in *the lower animals* a situation referable to such species differences as a form of antibody that is retained longer in the skin a marked cutaneous reactivity to histamine and the possession of a rich superficial lymphatic network The characteristic reaction *in the skin, the 'wheal and flare* consists of a "triple response' (1) an initial erythema owing to local vasodilatation of capillaries (2) a *flush or flare* spreading gradually from the center, owing to widespread arteriolar dilatation (local axon reflex), (3) development centrally of a "wheal' (a *sharply circumscribed elevated blanched* area sometimes showing pseudopodial extentions) The central whealing is referrable to the passage of fluid through the minute vessels owing to damage and increase of permeability The entire area involved may extend from 3 to 10 or more centimeters in diameter The reaction *occurs within 5 to 30 minutes* and usually begins to fade within a further 15 to 60 minutes after which the skin may regain its normal appearance

Sir Thomas Lewis (1927) showed that all three of the successive events could be reproduced by an intracutaneous injection of histamine into the human skin He brought evidence that a diffusible 'H substance and *perhaps other active materials* was liberated locally in the wake of cellular injury (owing to antigen antibody interaction or to direct injury by a variety of stimuli) His interpretation has not found acceptance everywhere (Ratner 1943) Evidence for the participation of histamine in allergic conditions of man has been sought repeatedly Transitory increases *have been detected in instances of physical* allergies (photosensitivity allergy to cold) and dermographia The failure to obtain more concrete evidence is not unexpected in view of the rapidity with which histamine can be re*moved from the blood* (Rose 1940) especially when only small amounts producing local symptoms are involved Obviously the situation is not yet clarified *substances other* than histamine may be involved at times

The Prausnitz Kuestner (P K) Reaction In individuals with clinical allergies such as hay fever and asthma to pollens and danders (and in some of those with food sensitivities) cutaneous reactivity did not coincide with a possession of ordinary antibodies their sera showed no precipitate with extracts of the *offending materials* and passive anaphylactic or cutaneous sensitization of the guinea pig although experienced now and then (de Besche 1923 Ramsdell 1930) was rare Finally however antibodies of a special sort

TABLE 15

	REAGIN	CLASSIC ANTIBODY	CO PRECIPITATING ANTIBODY	BLOCKING ANTIBODY
Heredity implicated	Yes (Atopics)	No	No	No
Experience with antigen	Natural (spontaneous) exposure	Via tissues	Via tissues	Via tissues
Protein type	γ_1 globulin (solely?)	γ globulins	γ_1 globulin	
Heating at 60 C	Labile	Stable	Stable	Stable
Reaction in vitro with antigen	None shown unequivocally	Precipitation	Adds to Antigen antibody precipitates	No precipitation Reaction shown by special method
Passive sensitization of skin	Fixes to skin (leads to positive P K test)	Leaves skin readily (no P K test)	Leaves skin readily (no P K test)	Leaves skin readily (no P K test)
Placental passage*	No	Yes		Yes

Review by Kuhns W J 1956 Types and distribution of antibodies Amer J Med 20 251 274

differing from the classic antibodies were detected by mean of passive transfer (Prausnitz and Kuestner 1921) These antibodies are termed reagins or less precisely skin sensitizing antibodies they are not to be confused with Wassermann reagin which has for long been a designation for the syphilitic antibody

Prausnitz received in his skin a small amount of serum taken from Kuestner who was markedly sensitive to fish and an extract of fish was injected 24 hours later directly into the site so prepared A striking wheal and flare reaction developed and faded rapidly Then this technic was extended largely through the detailed studies of Coca and Grove (1925) and was found to be applicable in many clinical allergies and to represent a powerful investigational tool Indeed in special cases of exquisite hypersensitivity diagnosis of the responsible allergen has been made preferentially by testing sites on a normal individual passively sensitized with patient s serum (As with all transfers of serum the chance of infection must be weighed)

The passively sensitized site will react when the allergen is introduced locally or when sufficient has been absorbed from the blood following injection of a larger amount into remote tissues (cf Lippard and Schmidt 1937) or following ingestion or inhalation (Walzer 1942)

The latent period between injection of serum and testing as practiced by Prausnitz and Kuestner is useful in securing a markedly increased sensitivity of the skin and in avoiding misinterpretation owing to toxic effects caused by certain sera (immediate whealing through liberation of endogenous histamine also other types of tissue irritation that may last for a few days) But at the cost of working with submaximal reactions allergen and reagin may be mixed and injected together into normal human skin to produce wheal and flare reactions

Reagins The antibodies demonstrated by Prausnitz and Kuestner in the serum of spontaneously allergic individuals exhibit certain special properties as shown in Table 15 That they are associated intimately with the gamma globulins of serum has been shown recently by column chromatography (Humphrey and Porter 1957 Sehon et al 1957) but their distribution is more heterogeneous than is observed with other antibodies *

The characteristics that distinguish reagins are these (1) They are unduly heat labile losing their tissue sensitizing function when

* Certain bizarre findings warrant much further study thus after a reaginic serum in a case of physical allergy to cold had been separated into several protein fractions successful transfer was possible only by use of a mixture of component fractions (Sherman and Seebohm 1950)

held at 56° C for between 30 minutes and 8 hours, or 60° C for ½ to 1 hour There is no critical temperature and the degree of heating required for full inactivation varies from one serum to another (Schmidt and Lippard 1937) (2) Reagins injected into normal human skin (and probably other tissues) are detectable up to 45 days (Lippard and Schmidt 1937), but at any time a reaction induced by allergen will discharge' the site and exhaust the reagin then remaining (3) Reagins do not appear to pass the placental barrier being absent in children born to allergic mothers (4) Reagins as found in natural cases of hypersensitivity do not precipitate the allergen and in mixture with allergen they do not fix complement (5) Reagin is found to be incapable of sensitizing guinea pigs to undergo anaphylactic shock

The development of reagins or the reaginic type of skin reactivity in a sample of the population has been observed several times under conditions of natural exposure e g, to dead sewage flies (Psychoda) (Ordman 1946) and to castor bean pomace (Figley and Elrod 1928 1950) The small proportion of reactors among those exposed is at least consonant with the idea of inherited susceptibility At the other extreme is the formation of the reagin type of antibody by the populace at large upon adequate artificial immunization with selected materials namely extracts of Ascaris (Rackemann and Stevens 1927 Davidson Baron and Walzer 1947) and of Trichina (Baron and Brunner 1942) and horse serum (page 167) Apparently in an intermediate position between these two groups stands the relatively high proportion—at least 40 per cent—of persons who develop flare and wheal reactions to diphtheria toxoid in consequence of a single booster dose of purified toxoid (Kuhns and Pappenheimer 1952) Such a production of reagins experimentally (and safely) should provide sufficient quantities of high titered sera to permit full experimental analysis

The sera of patients exhibiting physical allergies (toward light, or cold) have at times the ostensible features of reagins and may be examined by the Prausnitz Kuestner procedure

The reagins can sensitize passively not only human skin but the nasal and the ophthalmic mucous membranes and the mucous membranes of the intestinal tract, in studies on rhesus monkeys human reagins have been shown to sensitize the stomach (submucosa) and the gallbladder (lamina propria) as well The reactions induced in such prepared sites consisted of conspicuous edema development of pallor followed by hyperemia and hypersecretion In the passively sensitized monkey, as in the food sensitive human being administration of allergen in the diet resulted in pronounced gastric retention owing in part to occluding edema near the pylorus in the bowel there was either spasm or dilatation For a summary of these studies the reader is referred to Walzer (1941)

The hindrance involved in detecting reagins by passive transfer in human skin has prompted many attempts to determine it by other means There has been a difficulty in interpreting positive findings since occasionally reagins are associated with small amounts of nonreaginic antibody developed to the same allergen when the immunologic apparatus was stimulated (cf Thermostable Antibody p 171) Particularly with protein allergens—egg white, milk and epidermal antigens in particular—small amounts of antibodies are detectable in vitro or by anaphylactic shock of guinea pigs In this connection Vaughan and Kabat (1953) have pointed to the difficulty posed by impure test antigens a special allergen found by them in well recrystallized ovalbumin provided evidence that antibodies of more than one specificity may occur together and that positive effects may be attributed falsely to the system most readily measured

Using another protein system Kuhns and Pappenheimer (1951 1952) studied the flare and wheal reactivity associated with the presence of reagins that they observed to develop in a large proportion of persons developing diphtheria antitoxin during immunization with diphtheria toxoid (cf Neill and Fleming 1929) either rather well purified toxin or toxoid served as the allergen Most individuals who possessed reagin had formed the classic type of precipitating antitoxin others formed both precipitating and co precipitating antitoxin (Chap 5) but one or two formed almost only co precipitating antitoxin By use of the latter specimens they identified the reagin with co precipitating antitoxin However, questions regarding the toxin with re

spect to a content of other constituents have arisen (Kuhns 1957) If indeed co-precipitable antitoxin is the reagin it presents the unique feature of fixing readily in guinea pig skin and being then revealed by Passive Cutaneous Anaphylaxis quite unlike the typical reaginic sera encountered by the practicing allergist Further studies by Kuhns (1954, 1955) show that the skin sensitizing property of nonprecipitating antitoxin migrate largely as a γ_1 globulin (1954) but it has been found (Kuhns 1955 1956) that the skin sensitizing property is not a characteristic of all human nonprecipitating antitoxins

Cavelti (1950) using a delicate test has reported the presence of complement fixing antibodies in more than 75 per cent of patients allergic to epidermal antigens and in about one half of patients allergic to pollens

Quite recently Sehon Gordon and Rose (1957) have found certain sera from untreated cases of ragweed to agglutinate red cells to which ragweed allergen has been attached by means of chemical linkage through bis diazotized benzidine It is certain that blocking antibodies are detectable by this means it is now believed that reagins also react

Certain specimens of animal immune sera contain antibodies that fix in human skin just as with the human reagin they tend to be heat labile and are associated with faster moving components of serum than the classical precipitins (Aladjem et al 1957)

Thermostable Antibodies vs Reagins In desensitizing allergic individuals by repeated injections with tiny amounts of their allergens it was known that the ensuing clinical improvement was not related to a diminution in the amount of circulating reagins (Levine and Coca 1926) It was discovered later that the course of injections was giving rise to other thermostable antibodies that combine readily and specifically with the allergenic material (Cooke et al 1935 Loveless 1940) The thermostable antibody thereby neutralizes the latter so that a mixture of the two will fail to produce a positive skin test on a sensitive individual (Loveless 1940) and consequently it has been termed the blocking or inhibiting or neutralizing antibody Even normal individuals under the same treatment give rise to the same antibody but fail to produce reagin (Cooke Loveless and Stull 1937 Loveless 1942)

The characteristics of blocking antibody are given in Table 15 It withstands temperatures deleterious to reagins permitting its determination in allergen treated patients who possess both types of antibody Conversely, since blocking antibody readily leaves the skin after injection while reagin does not it is possible to measure reagin separately In order to test blocking antibody in vivo, mixtures made with allergen are injected directly into sensitive or passively sensitized skin The concentration of blocking antibody does not rise sufficiently high in patients possessing it to influence cutaneous testing with allergens

It may be noted that the practice of heating to differentiate among antibodies presents no clearly defined limits For example among the antibodies arising in cases of serum sickness not only those responsible for the skin sensitizing property but also those involved in in vitro precipitation and the establishment of passive anaphylaxis seem to be abolished by moderate heating (Sherman Cooke Crepea and Downing 1948) human diphtheric antitoxin subjected to similar heating loses its ability to precipitate with toxin but retains its antitoxic function (Cohn and Pappenheimer 1949)

Since thermostable antibody fails to precipitate the allergen its presence is measured by in vivo tests Recently with respect to blocking antibody for ragweed reagins several methods for detection in vitro have appeared and perhaps some may come to have practical usefulness agglutination of erythrocytes bearing chemically attached pollen solutes (Sehon Gordon and Rose 1957) agglutination of tanned erythrocytes having adsorbed pollen solutes (Feinberg and Flick 1956) participation usually as inhibitor in the ragweed extract rabbit antiragweed precipitating system The complexity of ragweed pollen with respect to its 7 to 15 allergens and the apparent need to recognize individual blocking antibodies would seem to require that separated allergen be available Stimulation of this second antibody adjusted like the reagin to the same allergenic material has been considered to be responsible for the clinical improvement and to be the aim of treatment This logical view is still contested chiefly on

the grounds that demonstrable antibody does not always accompany clinical improvement At present most test cases are too complicated to permit analysis ragweed extract, with its numerous soluble antigens, would have to produce, in desensitizing as many blocking antibodies as the patient has reagins! A more favorable situation for deciding the issue is offered by cases of insulin sensitivity coexisting reagin and thermostable antibody have been encountered, the thermostable antibody appearing indeed to be responsible for periods of insulin fastness' (Lowell 1944, 1947 Loveless 1946 Berson et al, 1956)

The existence of a pair of different antibodies both having similar serologic specificity is exemplified not only by reagin and thermostable blocking antibody but also (in serum sickness) apparently by reagin and precipitating antibody in the blood typing laboratories both Rh agglutinins and Rh blocking antibodies are encountered among human sera having the same specificity but differing from one another in heat stability in penetration through the placenta and in capacity to agglutinate Rh erythrocytes in salt solution (However the Rh blocking antibody acquires an agglutinating function when certain nonspecific protein materials are present in the diluent or when the surface of the red cells has been altered as by the action of trypsin) There is accordingly evident variety among the antibodies produced in the human being

The Relation of Allergic Insults to Human Disease

In the hope of elucidating the mechanism of certain diseases of man such as joint involvement rheumatic fever and periarteritis nodosa sensitized animals have been injected with antigen repeatedly and examined for evidence of acute and chronic pathologic alteration Alternatively, large doses of foreign serum (10 ml /Kg) or isolated protein (0 25 1 Gm /Kg) have been introduced into the blood stream of normal animals the protein being intended both to sensitize and by its persistence in the tissues to provide antigen for reaction with newly formed antibodies In a more recent line of inquiry normal animals have been injected with preformed antigen antibody complexes Positive evidence of vascular injury and associated edema and of duplicated human pathology, have been forthcoming giving further impetus to the idea that allergic insults are the cause of certain human diseases There would be one basic mechanism according to the concepts of Rich and others for periarteritis nodosa rheumatic fever, disseminated lupus erythematosus and rheumatoid arthritis Diseases of this class have been termed 'collagen diseases by Klemperer in view of the observed damage to collagen Whichever event would occur in a given human case would be referable primarily to individual predisposition and the peculiarly elective sites in which either the antigen becomes fixed or antibody is produced In view of the relatively limited ways in which tissues can respond to various stimuli (Klemperer 1947), each instance must be examined on its merits in many human diseases, antigenic complexes remain conjectural and the presence of antibody is undisclosed

The effect of repeated injections of soluble antigens into sensitized animals has been studied by many workers Longcope (1913) and Boughton (1916 1917, 1919) were able to produce myocarditis, periportal cirrhosis of *the liver widespread subacute nephritis and* subacute damage of the small arterial vessels while McLean et al, 1951, induced a diffuse glomerulonephritis in rabbits by repeated small intravenous injections of horse serum More severe changes owing to the injection of large amounts of antigen into well sensitized rabbits have been studied by many workers (cf Germuth 1953), definite injury being seen at times in myocardial ves els as well as acute and chronic glomerular lesions Junghans (Klinge 1933) encountered even granulomas of the coronary vessels aortitis and endocarditis

Localized inflammatory responses may be caused in animals possessing a generalized sensitivity as by irritating the skin with xylol or heating or chilling such procedures permit circulating antigen to induce local lesions in sensitized rabbits (Opie, 1936 Klinge 1933) *Intense local reactions may be induced also* by introducing antigen in high local concentrations (knee joints arterial walls and the like) Violent allergic inflammation in consequence of injecting antigen into the knee joints of sensitized rabbits was experienced by Klinge (1933) who found intense inflammation in

the adjacent soft tissue with necrosis and collagenous degeneration when these injections were made repeatedly into the same joint a severe destructive and deforming arthritis occurred Hartley and Lushbaugh (1942) secured extensive necrosis of the liver parenchyma by injecting specific antigen via the mesenteric vein Likewise by introducing antigen into the foot of the guinea pig 3 or 4 days before the onset of sensitivity—itself established principally from a distant repository of the same antigen in a water in oil emulsion—Kopeloff and Kopeloff (1949) have been able to produce a most striking inflammatory reaction leading to a chronic arthritic deformity of the foot And unilateral glomerulonephritis has appeared in sensitized rabbits following the injection of bacterial cells directly into the renal artery (Lukens and Longcope 1931) evidently because of a local *retention of bacillary antigen in high concentration*

A further advance was made when Rich (1942 1945 1947) studied the changes in human beings in consequence of treatment with antigens In the organs of patients acquiring fulminating serum disease following the administration of foreign serum *and* sulfonamide or iodine medication fresh vascular lesions characteristic of periarteritis nodosa were found Biopsies of testicular tissue secured before and after the onset of serum disease made it highly probable that these lesions were a manifestation of the hypersensitivity Then returning to the technic of giving rabbits large initial intravenous doses of horse serum (10 ml/Kg) and sacrificing them between 1 and 7 days after the last injection Rich and Gregory (1943) were able to demonstrate similar arterial lesions (and also acute diffuse glomerulonephritis) From such studies of rabbits made by Rich and his co workers and by many others afterward it is clear that the gamut of allergic changes includes acute necrotizing arteritis (Fig 20) endocarditis and subendothelial cellular infiltration glomerulonephritis of several degrees of severity and granulomatous lesions in the spleen and the lymph nodes Cardiac vascular and renal pathologic alterations are similar morphologically to those of rheumatic fever or rheumatoid arthritis acute glomerulonephritis and polyarteritis nodosa with associated granulomas



Fig 20 Section through submucosa of stomach of rabbit subjected to allergic insult showing marked necrosis of medium sized arteries An oblique section of an artery has been exposed revealing two segments of its lumen Marked localized damage is evident There is slight infiltration of polymorphonuclear leukocytes in and about the necrotic arterioles Hematoxylin and eosin stain × 75 The histologic finding is typical this particular animal was prepared passively by the slow infusion of antibody in the presence of antigen (Germuth F G Jr and Pollack A D 1958 Bull Johns Hopkins Hospital *5* 245 26? Photograph from Dr F G Germuth Jr)

Hawn and Janeway (1947) working in the manner of Rich administered to rabbits isolated beef gamma globulin and albumin and found that the times required for lesions to *develop varied according to the inciting pro*tein They were similar to the respective incubation periods observed before onset of the human serum sickness induced by these two materials—lesions due to albumin were maximal in 2 weeks those due to globulin were more pronounced at 1 week than later Acute lesions were found only when antigen was still present in the circulation while healing and healed lesions were to be seen only after antibody had appeared in the serum

Germuth (1953 Germuth Flanagan and Montenegro 1957) made a careful study of the time of appearance of lesions in relation to the production of antibody With bovine albumin (Fig 21) glomerulonephritis arteritis and reversible granulomatous lesions in the spleen and the lymph nodes were found to ap

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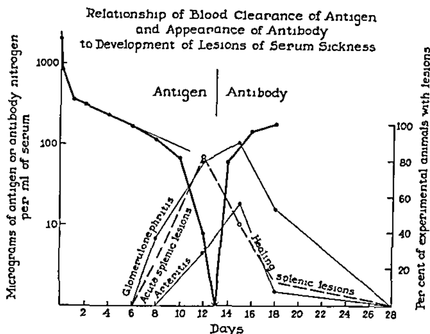


FIG 21 (Germuth F G Jr 1953 A comparative histologic and immunologic study in rabbits of induced hypersensitivity of the serum sickness type J Exper Med *97* 257)

pear only in animals that responded with early antibody production and only during the phase of so called immune clearance of circulating antigen The minimal amount of antibody required to produce this degree of immune clearance was estimated as 25 to 40 mg of antibody nitrogen with the proportion of antigen and antibody associated with production of single lesions of the vessels remaining unknown Upon the appearance of free antibody the various lesions shortly resolved the histologic findings of different workers obviously had varied with the time of sacrifice The differences that Hawn and Janeway had noted between the antigens—albumin produced arterial lesions gamma globulin chiefly kidney and nonarterial heart lesions—were shown to be related to the differential rates of catabolism of these proteins and to disappear largely when globulin was administered more than a single time (Heptinstall and Germuth 1957)

It will be evident that this laboratory guidance into the principles of tissue damage requires further experimental elaboration before the variety *among the so-called* ' collagen diseases of man can be understood The fact that the affected rabbit is apt to recover upon production of antibody might be contrasted with man s less well developed ability to form antibody or with the superiority of effecting chronic tissue damage by using a sequence of streptococcal antigens in rotation

In a proportion of rabbits subjected to a series of intracutaneous infections over a long period of time Murphy and Swift (1949 1950) induced successive infections with different serologic types of Group A streptococci cardiac lesions were found that resembled closely those of human rheumatic fever, including Aschoff bodies, and appeared to differ in several respects from the lesions induced by large amounts of foreign serum streptococci were chosen because of the long suspected association between rheumatic fever and preceding streptococcal infection Genetic factors appeared to determine the proportion of the rabbits that will become thus affected

The apparent requirement that anaphylactic reactivity in man must be prominent at times only in particular sites has led to a search for analogous situations in animals Evidence for local antibody formation is now well established A local area of sensitiveness has been produced experimentally in the rabbit s eye by Von Szily and by Seegal and Seegal (1931), the eye sensitized by previous

injection of antigen into the anterior chamber exhibiting hyperemia edema and lacrimation upon a later intravenous injection of specific antigen and even sometimes (with a time lag) upon introducing the antigen by stomach tube Experiments of this sort have become comprehensible with the newer evidence of local antibody formation (cf Batty and Warrack 1955 White, Coons and Connolly 1955) including the connection between antibody formation and plasma cells Also special roles can be credited to local concentrations of antigen maintained during the development of antibody Placing antigen in the pericardial sac of the normal rabbit proved to be more regularly effective than other routes in engendering a sensitization affecting the coronary vessels of the heart (Seegal and Wilcox) and local lesions in rabbit stomach could be produced in the same way (Jabiel et al 1952) Apparently rather similar in principle is the onset of experimental glomerulonephritis that is induced by administering low titer specific antikidney antibodies made in other species In contrast with the nearly immediate onset of kidney damage that follows the injection of first quality immune sera (cf Hasson Bevans and Seegal 1957 Seegal and Bevans 1957) such sera in diluted form or sera possessing lower concentrations of antibody require a latent period of 4 to 12 days As Kay (1940 1942) pointed out in explanation instead of injuring the kidney of the recipient directly the foreign gamma globulin probably remains largely on the renal tissue for which it possesses specific affinity constituting there a local concentration of foreign antigenic protein (cf Pressman Korngold and Heymann 1953) Once *initiated* the acute glomerulonephritis can prove to be fatal within a few days or can undergo remission for several months to be followed by chronic nephritis that persists for months or years The reason for persisting *nephritis is not yet understood*

Auto Antibodies in Allergy

The suspicion has long been entertained that antibodies might arise to one s own tissues (*perchance altered* through disease) and react with cytotoxic effect As one prototype there was the auto antibody responsible for paroxysmal hemoglobinuria acting on the host s erythrocytes but only at subnormal body temperatures Accordingly auto antibodies have been postulated to be responsible for diseases such as glomerulonephritis and perhap rheumatic fever it is necessary to distinguish auto antibodies as such from antibodies which may be provoked by complexes that can arise when certain exogenous materials (bacterial products, drugs) couple to body proteins or cells and impose a special antigenicity on the latter Evidence is accumulating in several diseases by use of fluorescing antihuman gamma globulin antibody (cf Coons 1956) that the patient s own gamma globulin affixes in sites of specific lesions—systemic lupus erythematosus and nephrotic glomerulonephritis (Mellors Ortega and Holman 1957) subcutaneous nodules of rheumatoid arthritis and heart valves in fatal rheumatic fever (Vazquez and Dixon 1956) the possibility that the gamma globulin represents auto antibody is under intensive investigation

That a rabbit can respond with antibody formation to its own sperm was shown long ago by Metalnikoff A more apposite experimental model was seen when Burky (1931) reported that staphylococcal toxin would enhance the low grade antigenicity of beef lens protein in the rabbit and cause rabbits to produce antibodies to lens protein and undergo allergic damage in the rabbit s own eye following deliberate lens injury However rabbit lens employed similarly was of minor consequence only (Swift and Schultz 1936) At about this time Schwentker and Rivers (1934) showed that monkey brain—usually altered by autolysis or viral infection—was antigenic in monkeys their work led directly to experiments that resulted in production of *iso-allergic encephalomyelitis which will be* discussed later along with other debatable instances of auto antibodies inasmuch as circulating antibodies do not appear to play a causal role

Various other reports not yet established have appeared antigenicity of homologous kidney combined with staphylotoxin as adjuvant (Schwentker and Comploier 1939) glomerulonephritis and cardiac lesions produced *in rats by injection of corresponding homolo*gous organs (kidney or heart muscle) mixed with killed group A streptococci and also the appearance of labile antibodies reacting with the re pective normal organs (Cavelti and Cavelti 1945 Cavelti 1947)

Of major importance has been the finding by Witebsky and Rose (1956) that homologous thyroid is antigenic in rabbits when injected with mycobacterial adjuvant and gives rise to antithyroid antibodies and specific lesions of the thyroid gland a rabbit can be immunized even by a part of its own thyroid Similarly Roitt Doniach Campbell and Hudson (1956) have detected in human beings affected with lymphadenoid goiter antibodies reacting with human thyroglobulin

The origin and the significance (in terms of allergic episodes) of certain other important factors found in the serum of patients have not yet been determined Patients with lupus erythematosus (L E) possess a gamma globulin which is responsible for the formation of the bizarre L E cell that appears in the blood the factor fixing to the nuclei (Miescher et al 1954 Holman and Kunkel 1957) of polymorphonuclear leukocytes which then form a swollen body become free and undergo ingestion by normal polymorphonuclear leukocytes Although the factor fixes to the separated nuclei of several species the possibility is raised that the L E factor might be viewed as an auto antibody to nucleoprotein or deoxyribosenucleic acid (cf Robbins et al 1957) Also certain patients with rheumatoid arthritis develop a special high molecular weight gamma globulin (having a sedimentation constant of 19s) that has strong affinity for the usual 7s antibody globulins of both man and rabbit and often forms aggregates averaging 22s (Franklin et al 1957) hence it can be demonstrated by several special in vitro tests e g agglutination of sheep cells that are sensitized with a subagglutinating amount of antibody by flocculation of latex particles that are coated with human gamma globulin or precipitation of heat aggregated normal gamma globulin

A plasma factor has been demonstrated in patients with thrombocytopenic purpura leading to a marked and prompt reduction in the numbers of platelets in transfused normal volunteers and producing frankly hemorrhagic manifestations in some of them (cf Harrington Minnich Hollingsworth and Moore 1951 Harrington Minnich and Arimura 1956) the activity is found to reside in the globulin fraction (Evans et al 1951) However, the antibody is often complex in its origin the development of idiopathic thrombocytopenic purpura is frequently related to administration of allergenic drugs or foodstuffs A notable advance was made by Ackroyd (1949, 1955) in studying thrombocytopenic purpura that followed ingestion of the drug sedormid an antibody was detected that agglutinated fixed complement and evidently lysed the patient s platelets whenever the drug was ingested In vitro studies on the resulting antibody drug platelet complex have been possible in both sedormid and quinidine hypersensitivities (Ackroyd 1955 Bolton 1956 Shulman 1957) Such instances are not "auto allergies in the full sense since the antibody arises from the stimulus of some altered drug tissue complex

ALLERGIC INFLAMMATION DELAYED RESPONSES

We shall now consider allergic reactions that require some hours to become manifest after the test material is deposited in or on the tissues These reactions are set apart not simply because they develop more slowly than do those of the 'early variety but because they exhibit no readily demonstrable relation to circulating antibodies and the procedure of passive transfer by means of serum is typically unsuccessful However transfer with white cells or cellular lysates has been demonstrated in several key ' instances without evidence for a gamma globulin type antibody (Table 13) Analysis has been further confused by the coexistence in some cases of both an early and a delayed type of response

Delayed responses as seen in the skin may involve epidermal cells alone or the deeper tissue layers according to the manner of sensitization and consequently various dermatologic classifications have been erected (Sulzberger 1940) Epidermal sensitivity is induced chiefly by contact of the skin with substances of low molecular weight (drugs nickel salts urushiol from the poison ivy plant and the like) or with products of fungi upon a subsequent contact of the same or a chemically related agent epidermal reactions of eczematous type appear with such lesions as macules papules or vesicles and with hyperemia and itching there is evident spongiosis in the tissues A sensitiveness apparent in the deeper layers of the skin results from invasion of the body by a variety of infective agents (bacteria fungi parasites viruses etc) or from the

use of drugs In the case of so called allergy of infection usually termed simply bacterial allergy the sensitivity is detected by injecting various extractives or metabolites of the infectious agent (or entire viral suspensions) into the skin (cf Jadassohn 1932)

A basic relationship is coming to light between quite different manifestations of certain dermatitides such as contact dermatitis and the tuberculin type of allergy and it seems entirely probable that a common cellular mechanism underlies such various manifestations

The hypersensitive state related to infectious processes will be considered first Indeed the outstanding example of reactions of delayed type was provided by Koch s discovery in 1891 of the tuberculin tuberculosis relationship The underlying allergic basis was not recognized until 1903 (von Pirquet) and because of several confusing factors a clear distinction between the tuberculin reaction and reactions of the anaphylactic type seems to have been drawn only much later (Coca 1920 Calmette 1920 Zinsser 1921) The tuberculin tuberculosis relationship is so fundamental that it will be presented in detail

Tuberculin Hypersensitivity

Koch s experiments with guinea pigs that had been rendered tuberculous showed plainly that infected animals possessed a special reactivity toward reinjection of the bacterium if the infection had been established for 2 weeks or more Upon attempted superinfection by the subcutaneous route there occurred an unexpected and massive inflammatory reaction of the tissues which walled off the injection depot and usually led to slough—the Koch phenomenon Since this reaction did not require living cells Koch sought a bacterial extract possessing the same property and attained it by heating the mycobacteria for some hours in the medium in which they had grown (meanwhile concentrating the fluid) and finally removing the bacterial residue This preparation was called O T or old tuberculin When this product was injected subcutaneously it was absorbed rapidly and caused a severe to lethal shock of delayed type termed the systemic reaction Upon necropsy *local* and *focal* reactions were found locally along the needle track and in the subcutaneous depot there were hemorrhage and pronounced edema the tissues appearing bluish red and rather gelatinous and the draining lymph nodes enlarged and discolored there was hemorrhagic exudate in serous cavities and everywhere the existing tuberculous lesions exhibited focal inflammatory reactions with enormously dilated capillaries and dense leukocytic infiltrations The focal reactions seem to give rise to the general toxemia and death

In 1903 von Pirquet advanced the hypothesis that tuberculin shock was another of the phenomena of sensitization and this belief led him to the discovery in 1907 of the *cutaneous tuberculin reaction* so important diagnostically

A delayed type of *ocular reactivity* was noted almost simultaneously Indeed the three types of response remain as our most stringent criteria of delayed type hypersensitivity to water soluble allergens but such critical tests are possible only in sensitized laboratory animals *local* reactions at the site of skin tests showing induration at 48 hours *systemic* reaction to injections made by the peritoneal or intravenous routes and *corneal* reactions resulting from intracorneal injection Even in diagnostic testing by intracutaneous injection (Mantoux) it is necessary to select the dosage of tuberculin with caution for an excess of tuberculin may cause not only unduly severe reactions in the skin but also some degree of lighting up around tuberculous lesions and febrile systemic reactions (Tytler 1930)

Obviously bacteria offer numerous antigens to the invaded host In the case of *Myco tuberculosis* circulating antibodies to perhaps 7 of these have been discovered by the application of special technics (Boyden and Sorkin 1956) although the concentration of the antibodies is seldom high Indeed one of the early difficulties in interpreting the tuberculin reaction had to do with the occasional demonstration of an anaphylactic state in tuberculous guinea pigs It is now rather well agreed that these antibodies are not causally related to the delayed type hypersensitivity Several of the tuberculoproteins tested in their native state elicit cutaneous delayed type reactions one being heat coagulable and of large molecular size (ca 44 000) For the purpose of human skin testing it would seem to be desirable to elect low molecular and even heat

altered material, so long as it is serologically reactive, in order to avoid the acquisition of sensitiveness to proteins through repeated skin testing Present practice, however, is to prepare the testing materials such as PPD (purified protein derivative) from culture filtrates that approach the native state

When suitably diluted tuberculin is injected into the skin of the tuberculous individual, there is no obvious immediate reaction After a few hours redness appears at the injected site and the local inflammation with its associated edema gradually increases in intensity and extent for from 15 to 48 hours and attains a typical firm induration meanwhile, when the dose and the degree of sensitivity allow a more severe reaction there will have been central blanching and a gradual development of an innermost livid zone, which often becomes necrotic The inflammation then slowly fades but the lesion is palpable for some days and pigmentation may be seen for several weeks

The tuberculin type of response has been studied histologically by Dienes and Mallory 1932 Laporte 1934 Gell and Hinde 1951 and others While the anaphylactic and the Arthus types are characterized by primary local injury of blood vessels early development of edema a marked infiltration of polymorphonuclear leukocytes as the chief invading cell and relative paucity of mononuclear cells the reaction to tuberculin is attended after some hours supervening upon an initial more or less intense and general infiltration of polymorphonuclear leukocytes by characteristic and strong focal accumulations of mononuclear cells The special features of the tuberculin reaction are rendered even more evident upon injecting tubercle bacilli into the skin of normal and tuberculous guinea pigs (Dienes) Gell and Hinde (1951 1954) have studied reactions to tuberculin in the rabbit in view of the proliferation and the differentiation of the infiltrating monocytes and especially of the local histiocytes and undifferentiated mesenchymal cells within the first 24 hours it is suggested that there is constituted a relatively durable structure ' which may justifiably be called reticuloendothelial tissue These workers point to the presence of complex cellular "patterns' and are less inclined than others to distinguish between Arthus and purely ' tuberculin reactions When the tuberculin reaction is inten e it engenders necrosis requiring several weeks for elimination and repair Consequently, in later stages of severe tuberculin and Arthus reactions there are no differentiating histologic features

Typical but low grade tuberculin sensitivity can be induced by the injection of killed mycobacterial cells (Zinsser and Petroff, 1924 Flahiff, 1939), there is remarkable enhancement of sensitization when the dead bacilli are suspended in hydrocarbons such as paraffin oil (Saenz, 1935) In sensitizations effected with the use of hydrocarbons, accompanying anaphylactic manifestations are encountered more often than is seen in infection Many attempts have been made to obtain a bacillary extract capable of substituting for the whole killed cells in inducing the delayed type of hypersensitivity, for separated tuberculoproteins give rise to only the anaphylactic and the Arthus types of reactivity Finally Raffel announced that a wax fraction of the cells, in conjunction with tuberculoprotein present as an impurity or intentionally admixed possessed the property sought (cf Myrvik and Weiser 1952) The "directive effect of the wax in inducing sensitization to tuberculin appears to be dependent upon its lipopolysaccharide Choucroun (1939 1947) separated a lipopolysaccharide that gives rise to delayed type hypersensitivity while Asselineau and Lederer (1949) isolated a wax termed "D from human tubercle bacilli which in bovine strains remains bound to the cell walls it both sensitizes to tuberculin and acts as adjuvant Wax D of molecular weight 15 000 to 20 000 contains mycolic acid a polysaccharide composed of arabinose mannose and galactose and a peptide containing alanine glutaric acid and α ϵ diaminopimelic acid

Desensitization of tuberculin reactivity is seen, usually incompletely in tuberculous men and animals after a single large injection of tuberculin has produced severe constitutional reactions Desensitization is considerably more difficult in tuberculin sensitivity (and peculiarly hazardous because of the danger of focal reactions) than it proves to be in anaphylaxis But as Rich (1951) says desensitization in man by repeated, gradually increasing doses of tuberculoprotein has often been carried to a point at which the patients were able to tolerate without a focal or con

stitutional reaction doses of the protein which in the undesensitized body would undoubtedly have produced extreme focal reactions and death The desensitization of tuberculous guinea pigs is a difficult and laborious task requiring large repeated doses of the tuberculin preparation PPD but during th treatments the skin is maintained truly nonreactive and it even will react to intradermal spread of dyes in the same way as does normal skin (Birkhaug and Berle 1945) however sensitivity returns when administration is discontinued It may be added as emphasized by Rich that both in the occurrence of specific temporary desensitization and in the accelerated rate at which tuberculin hypersensitivity has been observed to reappear upon a subsequent reinfection there is a marked resemblance to the known behavior of the antibody producing mechanism these features have been cited as evidence that an antigen antibody interaction underlies the effect of tuberculin on sensitive tissues

Sensitivity to tuberculin appears not to be widespread among the cells of the tuberculous animal as was formerly believed Sensitivity of macrophages is evident even in tissue culture in migrating individual cells and apparently in their descendent or daughter cells at least of the immediately succeeding generations (Rich and Lewis 1928 1932 Aronson 1931 Moen and Swift 1936 Moen 1936c Heilman 1944 Waksman 1953) but the sensitivity is trivial with tissue fibroblasts and is not manifest in cultures of corneal epithelial cells or cutaneous or hepatic epithelium (cf May and Weiser 1956)

As Rich and Lewis observed in their classic paper (1932) when proper concentrations of tuberculin are added the damage done to allergic cells by tuberculoprotein is marked and easy to observe It is evidenced by a decided inhibition of migration of the allergic cells from the explant into the surrounding tuberculin containing plasma and by the fact that the relatively few cells which do wander out die in a few hours Macrophages fibroblastic growths developing from mononuclear exudative cells and granulocytes of infected guinea pigs and rabbits are found to be sensitive the injury to the migrating cells appearing to be cumulative over a period of some hours Whether or not the cells of human beings acquire comparable degrees of hypersensitivity is apparently open to question (Florio et al 1958)

In the intact animal the relatively avascular cornea of tuberculous guinea pigs reacts to direct injection of tuberculin (Holley 1935 Rich and Follis 1940) with edema swelling of the fibers infiltration with granulocytes and necrotization of corneal cells It may be mentioned as one differentiating feature that no similar type of cellular reactivity to injection of antigen has been obtained in the Arthus sensitized rabbit (Rich and Follis 1940)

Most important results have followed the finding that the delayed type of reaction to tuberculin can be transferred to a fresh animal by using washed cells of tuberculin sensitive donors Living white cells obtained from peritoneal exudates spleen lymph nodes or blood sufficed upon injection into a new guinea pig to cause it to acquire a hypersensitivity to tuberculin for a limited period of time but only if the cells had undergone no damage (Chase 1945) Kirchheimer and Weiser (1947) showed further that a second characteristic of bacterial allergy the systemic reaction to tuberculin is transferable by means of cells (cf Kirchheimer et al 1949 Wesslen 1952) It is highly probable that actual transfers were encountered in older attempts since tissue and white cells were often included inadvertently in the material transferred (cf Fellner 1919) the experiments were discounted because of wanting reproducibility

By means of the same technic of transfer using white cells collected from peripheral blood Lawrence (1949) accomplished the transfer of tuberculin sensitivity from man to man and (1952) streptococcal hypersensitivities likewise Indeed it proved to be far easier to provoke sensitization in man by means of cells Then unexpectedly Lawrence found that lysates of the cells would induce sensitization and this even after exposure to the enzymes deoxyribonuclease and ribonuclease Because the induced sensitivity was found to persist for many months a concept of a transfer factor was developed to explain the retained sensitivity in later cell generations Finally evidence was secured that pre incubation of cells with tuberculin would liberate

transfer factor' from the cells without undergoing visible damage The technic is delicate and has not led to identification of the active material

Somewhat parallel experiments with animal cells emphasize the early participation of the cells that are transferred Metaxas and Metaxas Buhler (1948 1949) found that the local deposition of the "transfer' cells in the skin of normal guinea pigs would give rise to local tuberculin reactions (probably with participation of a Shwartzman effect) if large amounts of tuberculin were administered parenterally Wesslen (1952) accomplished the same result with rabbits using lymphocytes harvested from the thoracic duct Finally Metaxas and Metaxas Buhler (1955) showed that there was no latent period between injection of the donated cells and establishment of the capacity of the recipient to react mixtures of cells and tuberculin were fully effective when injected intradermally These workers were unable to detect a more permanent phase of sensitivity such as Lawrence found in man Perhaps an indication of active soluble products analogous to transfer factor was provided by experiments on the transfer of bacterial allergy from brucella infected guinea pigs Carrere and Quatrefages (1952) reported that tissue mash mixed with melitine (the skin testing material) would induce local reactions upon injection into the skin after the mixture had been incubated for some hours soluble material could be harvested from the site of a positive reaction that would induce a positive reaction in still another normal guinea pig

Quite another line of investigation was opened by Favour (1947) who studied the effect of tuberculin on mature lymphocytes from tuberculous mice and guinea pigs In contrast with the slowly progressing damage observed by Rich in tissue culture when tuberculin was added a decrease of between one fifth and one half in number of intact lymphocytes was apparent in 1 hour or less after tuberculin was added Favour termed the phenomenon lympholysis It was found that even normal lymphocytes would undergo fairly prompt dissolution in the presence of tuberculous plasma complement and tuberculin and it appeared that the factor present in tuberculous plasma would be released into normal plasma by tuberculin sensitive lymphocytes (Miller and Favour 1951) the factor like an antibody, was precipitable with the serum euglobulin Whether the factor occurring in tuberculous plasma is an antisubstance of novel nature is to be examined closely in view of the several antibodies that have been recognized recently in tuberculous plasma (Boyden and Sorkin 1956) and the knowledge that antibody can be present in traces in or on small mononuclear cells (cf Harris 1951) Waksman (1953) reports that rabbit polymorphonuclear cells suffer damage under the same conditions employed by Favour for "lympholysis,' the effective antibody being apparently of the 'univalent variety Indeed the relation of lympholysis to the problem of dermal hypersensitivity is still obscure since tuberculous plasma quite active in this regard does not commonly transfer delayed type reactivity to tuberculin indeed Waksman (1953) states that dermal reactivity of guinea pigs to tuberculin shows correlation with positive inhibition of macrophages in tissue culture but not with 'lympholysis

Of greater moment is the report by Cole and Favour (1955) that adequately large amounts of plasma from tuberculin sensitive guinea pigs (e g 80 ml) can passively transfer delayed type dermal hypersensitivity after certain antibody containing fractions have been discarded If the report is substantiated the way should be open to examine the active but dilute serum borne principle perhaps transfer factor of Lawrence (*vide supra*)

Allergy in Microbial Diseases Other than Tuberculosis

As mentioned in other microbial infections (with bacteria fungi viruses) and in certain parasitic infestations there is exhibited much the same sort of delayed type reactions when the corresponding agents or extracts thereof are put into the skin In variable degree one finds also the other chief attributes of delayed type sensitization—systemic reactions sensitivity of the cells in tissue culture toward the respective allergens and characteristic damaging reactions upon intracorneal injection (to be sought as mentioned above only in experimental animals) Many of the e reactions are less pronounced than in the tuberculin tuberculosis relationship for as Boyd has remarked

tuberculin allergy is one of the more extreme examples of bacterial allergy Furthermore one finds the early types of sensitivity far more frequently the extracts employed for testing sometimes giving immediate type re actions particularly in relation to a content of specific polysaccharides Often both type of sensitization coexist

The degree to which microbial allergies may shape the disease pattern has been long de bated and we shall attempt no answer Judg ment must be reserved even on the long nurtured idea that an individual may owe his allergic difficulties (e g intrinsic asthma) to a sensitiveness to products of bacterial strains harbored within some focus in his own body (i e antrum root canal or socket of tooth Bartholin gland throat) Despite the convictions of some shrewd physicians there is no absolute supportive proof The process could well be at play in streptococcal pyoderma of children Cooke (1947) has cited scattered cases of infective asthma and of re curring intrinsic dermatitis in which the in jection of autogenous vaccine precipitated asthmatic crises or exacerbations and in which intervention by physical means or antibacte rial agents appeared to have been curative But skin tests with vaccines made from au togenous culture are usually not selective in indicating a prime allergen

Streptococcal Allergy Evidence of infec tion with streptococci is usually sought in the form of circulating antibodies directed against streptococcal enzymes and type specific anti gens However as with tuberculous infection but less sharply defined preceding infection can be witnessed by delayed type allergy to ward streptococci Relatively large samples of the population have been tested by means of killed streptococcal cells (vaccines) cellular substances (nucleoprotein M substance) and culture filtrates Employing culture filtrates of group A streptococci (human pathogenic forms) MacKenzie and Hanger (1927) found the incidence of sensitivity to increase grad ually with age and superimposed upon this a sharp rise in the number of reactors when ever diseases intimately associated with strep tococci became prevalent Recently Lawrence (1952) tested 472 persons having no apparent strepto occal infection or sequelae with one or another test substance and found that only about 11 per cent failed to react at all but that a well-established degree of sensitivity was confined to approximately half of the sub jects Further the sensitivity was expressed unequally against his testing materials sug gesting elective sensitization to different bacil lary constituents—vaccine cellular M sub stance and a partially purified culture filtrate rich in the enzymes streptokinase and strepto dornase

Allergy to streptococci has been well studied in laboratory animals In natural infections with group C streptococci there is pronounced skin reactivity to streptococcal extracts in guinea pigs that are ill with epizootic lymph adenitis or are healthy carriers of the respon sible micro organism (Moen 1936b) and reactivity in rabbits to extracts of their natu ral pathogen *Past cuniculicida* the organism causing snuffles (Hanger 1927) Both guinea pigs and rabbits can be sensitized at will

It was shown by Derick and Swift (1929) that rabbits could be sensitized with *viridans* streptococci by intracutaneous injection and by several other routes with the notable ex ception of intravenous injection In the latter case a type of immunity became established (at least to the homologous strain cf Ange vine 1943) but otherwise and apparently in connection with the development of some focal tissue reaction the animals presented cutaneous ophthalmic and systemic hyper sensitivity to the administration of living streptococci all being expressions of a delayed type of reaction unrelated to the amount or type of circulating antibody and not trans ferable by serum When rabbits thus sensitized were tested with the separated nucleoprotein or polysaccharide fractions of the cocci (Mc Ewen and Swift 1935) tuberculin type re sponses were secured but in addition the reaction to the nucleoprotein started as an immediate response and had an edematous as well as an indurated character This appears to suggest a coexistence of both sorts of skin effects

To relate further the mechanisms of strep tococcal and tuberculin hypersensitiveness Moen (1936a) examined both sorts of sensi tivity in parallel in tissue culture using ex plants of tuberculous tissues *and explants of* the spleen of guinea pigs infected with a natu

ral streptococcal pathogen (group C hemolytic streptococci from guinea pig lymphadenitis) The presence of streptococcal extract led to a slowly developing specific toxic effect on the cells of streptococcus infected animals, quite similar to but quantitatively less than that of tuberculin on tuberculin sensitive cultures The streptococcus sensitive cells exhibited more unequal gradations of sensitivity than is seen in tuberculin sensitive cultures, some cells being killed rapidly others being only slightly inhibited Very much less evidence of persisting sensitivity was seen in subcultures of the streptococcus sensitive cells as compared with explants of tissues from tuberculous animals

As mentioned above Lawrence (1952) has transferred streptococcal hypersensitivity from man to man by means of peripheral blood leukocytes paralleling his experiences in transferring tuberculin hypersensitivity

Pneumococcal Allergy Pneumococcal allergy has been studied in rabbits in much the same way as has streptococcal hypersensitivity In rabbits immunized by deposition of vaccine in the skin one finds a delayed type of skin reactivity toward pneumococcal vaccine or nucleoprotein and a delayed type of ocular reactivity (Julianelle 1930 Harley 1935 1937) by means of serum the manifestation of reactivity to nucleoprotein is passively transferable and apparently is of Arthus type while neither the allergy to vaccine nor the eye reaction is so transferable

With both nonhemolytic streptococci and encapsulated pneumococci intravenous injection of intact cocci leads to the development of anticarbohydrate antibodies and to immediate type reactivity expressed toward the corresponding specific polysaccharide

During the course of pneumococcal pneumonia two types of specific skin reaction have been observed (Tillett and Francis 1929) one in response to the type specific capsular polysaccharide appearing at the onset of convalescence and consisting of an immediate reaction of urticarial type the other elicited by species specific nucleoprotein and being of the tuberculin type

In addition to these two type of reaction there is a third variety for a while during the illness patients exhibit a skin reaction chiefly of the delayed type, upon being tested with the somatic "C polysaccharide of pneumococci Uniquely the skin reactivity here is not based upon an antibody mechanism but involves a special protein which appears temporarily in the blood not only in pneumonia but in a variety of other acute infections This special protein termed the C reactive protein' because it reacts in vitro with pneumococcal somatic C polysaccharide is sharply differentiable by serologic means from any of the normal blood proteins Were it not for experimental study, the positive skin reaction might have been attributed to an antigen antibody interaction

Other Bacterial Allergies Practical information exists regarding diagnostic skin tests in other bacterial diseases but there is less theoretical understanding The greatest need is in diseases for which the causative organism is not to be isolated readily

In the natural infection of man and horses with glanders and in experimental infection of the guinea pig a delayed type of ocular reaction skin sensitivity and a systemic, general reaction (temperature rise) are expressed to *mallein* analogous to the tuberculosis tuberculin relationship Likewise, in the infections of cattle caused by *Myco pseudotuberculosis* (Johne s bacillus) there are skin reactions conjunctival reactions and generalized systemic reactions to johnin' a preparation made like tuberculin and variously purified a reaction occurs also with avian tuberculin Johnin may be standardized by intradermal tests on hamsters and guinea pigs sensitized (but evidently not infected) by means of living bacilli in conjunction with substances which have an adjuvant effect, for instance paraffin oil or calcium phosphate (McIntosh and Konst 1943)

Skin reactions have been employed to detect present or past infection in brucellosis (with brucellin a culture filtrate or 'brucellergin an actively antigenic nucleoprotein extract) and in tularemia with chemically treated vaccines (Foshay 1932) The skin reactivity appearing in brucellosis of the guinea pig has been transferred by means of white cells and shown to be immunologically distinct from cell transferred hypersensitivity to tuberculin (Metaxas Buhler 1952)

Lepromin an extract of human leprous tissue often provokes skin reactions in patients with tuberculoid leprosy but the range of its usefulness is not established

In special cases sensitivity can be found

with respect to antigenic bacterial toxins and toxoids as marketed—tetanus toxoid diphtheria toxoid This sensitivity can be directed toward the toxin molecule itself (and the formolized toxoid derived from it) or against other constituents present in some commercial preparations (e g bacillary protein peptones of the medium) For example there has been seen in about one fifth of young adults but infrequently in children, a tuberculin type of sensitivity to purified diphtheria toxin and toxoid (Pappenheimer 1948) This tuberculin type of response representing the so called false or pseudopositive Schick reaction lasts a day or so and then fades usually it can be differentiated from the true Schick reaction—a primary toxic effect of toxin on normal skin occurring whenever neutralizing antitoxin is absent—in that the true toxic effect appears slowly and develops increasingly during the course of 4 or 5 days (In addition as we have seen immediate type flare and wheal reactions occur in some individuals to the e same constituents of diphtheria toxin and toxoid)

Allergy to Fungi In fungal infections the usual skin reaction is of the delayed type (24 to 48 hour reaction) Highly u eful diagnostic results have been obtained in coccidioidomycosis with coccidioidin an extract of ground heated culture growth and evidence of general systemic reactions has been seen In histoplasmosis of man and of experimental animals reactions are obtained with *histoplasmin* (culture medium which has supported the growth of *H capsulatum* for several months) In trichophytosis various preparations of *trichophytin* an extract of triturated cultures with their metabolites have been used the common diagnostic reaction is of the delayed variety but a few subjects have shown also immediate urticarial reactions and corresponding reagins and in them skin testing has been known to bring on asthmatic attacks

Allergic Reactions to Viral Materials Evidence of hypersensitiveness of the delayed type was ob erved unknowingly by Jenner in 1798 with the virus of cowpox individuals rendered immune by inoculation with cowpox virus later responded to this or the closely related smallpox virus with a small local redness that appeared within 48 to 72 hours and then faded quickly This manner of response was interpreted as an allergy by von Pirquet and Schick in 1903 the role of hypersensitivity was convincingly demonstrated when Hooker (1929) injected heat inactivated virus into the skin of cowpox immunized individuals

The Frei test has been used diagnostically in cases of lymphogranuloma venereum (injection originally made with diluted and heat sterilized pus then with infected mouse brain and now performed with antigen obtained from chick embryo cultures) A skin test betokening past infection with mumps virus has been developed by Enders et al (1945 1946) for the assessment of immunity Preparations of the parotid glands of monkeys infected with monkey adapted virus were employed initially but viral material grown in the allantoic fluid of embryonated eggs is under preparation

Allergy in Parasitic Infestations In contrast with the above observations the form of allergic response that has received chief consideration in parasitic helminth infestations —for example schistosomiasis echinococcus disease filariasis trichiniasis and ascaris infestation—has been the immediate reactions of the wheal and erythema type found when the skin of the host is tested with extracts of the body substance of the same or related parasites usually antibodies can be demonstrated in the serum by serologic methods at the same time Asthma and rhinitis are sometimes elicited upon exposure to the specific agents While there is often an early reaction to hydatid fluid in echinococcus infestation it appears that the delayed type or *Casoni reaction* should be given chief diagnostic interpretation

In protozoan infestations skin tests have been less useful The only instance to be mentioned is leishmaniasis in which a tuberculin like reaction has been observed in skin tests made with an extract of cultured leishmania

Even as an aftermath of sensitization by insect bites delayed allergic reactions have b en demonstrated (Benson 1936) independently of early reactions (and corresponding reagins) which occur at times both varieties may be seen in the same individual

Evaluation of Skin Tests

It is not our purpose to enter into a discussion of the diagno tic usefulness of each sort of skin test but rather to stress one underlying principle To make any test practicable for diagnosis the purification and the proper concentration of the te t agent its toxicity for tissues the degree of correlation between preceding invasion and definite skin reactivity and the relative specificity of the reaction must all be established It must also be known

whether the material employed for skin testing can lead to active sensitization and vitiate future tests Even in the most studied case tuberculosis investigation does not cease In those instances in which both early and delayed *reactions can be shown it is important* to evaluate each type of reaction independently

In general when the method of testing is specific such tests have a significance similar to that of tuberculin a positive reaction denotes the occurrence of infection but gives no *generally reliable indication of current activity* It is most informative when the skin reactivity is known to have been acquired recently One must recall furthermore that in advanced stages of all diseases, including tuberculosis the skin may fail to react this condition is called anergy and has been considered a consequence of an ' exhaustion on the part of the tissue cells Also in intercurrent infection such as measles and chickenpox the tuberculous host may temporarily cease to give skin reactions to tuberculin Even a positive test which is specific may not always be of practical diagnostic value for example Sulzberger (1940) points out that interpretation of reactions to extract of monilia (oidiomycin) must be weighed against the well nigh universal exposure to various species of *monilia*

The Adjuvant Effect of Tubercle Bacilli Auto allergic Diseases

Certain special properties of *Myco tuberculosis* came to light when Dienes (1929) showed that the injection of protein antigens into a tuberculous focus led to a delayed type of hypersensitivity directed against the protein as well as the expected appearance of circulating antibody The induced tuberculin type *hypersensitiveness to egg protein as* Burnet (1948) has said therefore seems to be definitely related to the placing of the antigen in an inflammatory area in which histiocytes and lymphocytes predominate

Following the discovery by French workers that the sensitizing property of killed mycobacteria is greatly enhanced in the presence of hydrocarbons Freund and McDermott (1942) imitated Dienes injection of protein into a tuberculous focus by blending horse serum *and an emulsifier* in paraffin oil containing killed tubercle bacilli and injecting the emulsion Probably owing to the type of cellular response evoked by the liquid hydrocarbon and the mycobacterial substance, an intense cutaneous reactivity to horse serum of delayed type resulted At the same time the output of circulating antibody became exaggerated and was sustained for many months

Therefore in animals so prepared a high degree of Arthus type reactivity is superimposed on the original "Dienes effect ' Reference to either or both of these effects is variously intended when the term ' adjuvant effect of killed mycobacteria' is used but it is necessary to distinguish between the effects due to the added mycobacteria and the effectiveness of antigen dispersed in hydrocarbon (water in oil emulsion) without the incorporation of mycobacteria or special mycobacterial wax So far as the concentration of circulating antibody is at issue the mycobacteria can often be omitted from the emulsion but the "directive effect of the mycobacteria is needed in order to secure certain of the delayed type allergenic effects

When brain substance was incorporated in the Freund adjuvant, partly in order to find whether enhanced antigenicity would lead more readily to demyelinating disease of the sort that Rivers and Schwentker (1935) had *obtained with many injections of autolyzed* brain it was found that a fatal demyelinating disease was induced in monkeys with disseminated lesions in brain and cord (Morgan 1946 Kabat Wolf and Bezer 1946) The effect was secured only when mycobacteria were included in the emulsion The disease currently termed experimental allergic encephalomyelitis has been studied intensively in laboratory animals particularly in guinea pigs rabbits rats and certain strains of mice *it has been viewed as a possible* laboratory prototype of multiple sclerosis (see Kolb 1950) That the mechanism is independent of any subtle intraspecies antigenicity of brain substance has been nicely *shown* by Kabat et al (1949) who induced the condition in monkeys in response to a portion of their own brain substance removed in a prior operation The active material in brain resides chiefly in the white matter and has been thought variously to be 'proteolipide or collagenlike in nature

Iso antibodies to brain substance are found irregularly but there is no correlation between the e and the pre ence of lesions and the serum does not transfer the condition to normal animals (cf Waksman and Morrison 1951) Further Waksman and Morrison have found that the intracutaneous injection of homologous spinal cord with the Freund adjuvant in rabbits leads to a delayed type dermal sensitivity and a corneal sensitivity expressed toward homologous cord as test material An interpretation should await separation and chemical characterization of the active material for the finding cannot be generalized iso allergic encephalomyelitis arises in guinea pigs without an accompanying dermal reactivity when guinea pig brain is the inciting antigen Efforts to transfer the encephalomyelitis by means of white cells have not yet succeeded although transfer during parabiosis has occurred with rats (Lipton and Freund 1953) For literature see Alvord and Kies 1958 An allergic neuritis has been shown by Waksman and Adams (1955 1956) to occur as another distinct entity when peripheral nerve is used instead of brain suggesting immunologic differences in myelins from the two sources

When testicular tissue instead of brain is incorporated in the emulsion another but non fatal iso allergic condition arises namely a marked degeneration of sperm cells from spermatogonia to the mature sperm this condition can be initiated by injection of material from one of the animal s own testicles removed beforehand (Voisin et al 1951 Freund et al 1953 1955) The active antigen is heat stable and appears to be a polysaccharide or glycoprotein

Contact Dermatitis Drug Allergy

The manifestations of allergy to drugs and chemicals are manifold and can duplicate all the aspects of allergic reactions that we have been considering—anaphylaxis immediate type reactions and delayed reactions (Tabl 13) Among the facets of drug allergy are included such various forms as erum sickness arthralgia asthma flare and wheal reactions urticaria scarlatiniform and morbilliform rashes exfoliative dermatitis and many others It is rare to encounter many forms in one individual and apart from inherited predisposing factors in the subject himself the chief determining elements appear to be the mode of contact through which sensitization has arisen and especially (though seldom obviously) the chemical properties of the sensitizing material More commonly encountered by far is the delayed type of reaction which will concern us here The reader is referred to Sulzberger (1940) Landsteiner (1945) and Carr (1954) for reviews which deal with historical and theoretical aspects

The inciting agents range from drugs (u ually water soluble medicaments) given by mouth by injection or by topical application to various materials of simple constitution usually of much more limited solubilities that are encountered chiefly as contactants The last term covers a gamut of materials from the metal nickel to the catechol derivative of the poison ivy plant urushiol which causes the allergy known as poi on ivy dermatitis It is to be understood that we are not dealing with exaggerated sensitivity to established pharmacologic properties of the respective materials

The causative agents are not antigens as such but it appears that by combining with the tissues of the host (either directly or after some intermediary chemical alteration in vivo) they acquire antigenic capacity and function somewhat like the conjugated antigens of Landsteiner which arise when hapten structures become attached to indifferent foreign protein

According to this concept the newly formed complexes which might be termed *derivative antigens* would effect the sensitization and reactions would be elicited later whenever the same or a chemically related substance presented itself by the proper route

This working hypothesis derived from chemical and immunologic theory would have several corollaries The sensitizing contact should it give rise merely to antigenic complexes that are soluble and are readily absorbed probably would induce only an anaphylactic type sensitization just as we find when solutions of bacterial nucleoprotein are injected into animals It is more to be expected in order for the delayed type of reactivity to develop that the formation of the sensitizing complex will occur in relation to some focal tissue reaction

And further we could well expect that often both the delayed type of reaction and the

formation of anaphylactic antibody would be induced simultaneously, even if wholly disproportionately

Upon the eliciting contact it is likely that mere contact between sensitive skin and the small molecule itself—analogous to the in vitro procedure of mixing antibody and hapten—would not suffice to induce a reaction owing to the small molecular size of most of these allergens, but that a new combination of the allergen with proteins or other substances of the host would be a prerequisite From this point of view the opportunity for observing 'cross reactions' in making tests with a series of related chemicals would be limited to those compounds whose chemical properties permit their joining with constituents of the body

Experimental investigation may be said to date from 1896 when Jadassohn first published his careful observations and introduced the patch test as a diagnostic procedure in the study of contact dermatitis For many years such allergies were considered to represent individual and innate idiosyncrasies only a few people for example become sensitized to the primrose plant (*Primula obconica*) This idea was shown to be untenable when Bloch and Steiner Wourlisch (1926) pointed out that any individual could be rendered sensitive if one simply used a concentrated extract of *Primula* leaves and a sufficient number of applications Some persons became sensitive after one application others required several treatments and evidently the role played by heredity was reflected in this The same workers next showed (1930) that guinea pigs as well could be sensitized to *Primula* and Mayer (1931) demonstrated that like human beings guinea pigs could be made sensitive to paraphenylenediamine a compound often used in the dyeing of furs The way was now open for animal experimentation for the manifestations of dermatitis in man and in sensitized guinea pigs were analogous even if not identical

At the cost of historical balance we may simply summarize the chief results of experimentation (Landsteiner et al Sulzberger *Haxthausen Grolnick etc*) Some of the chemicals that sensitize the human being will induce sensitivity in the guinea pig (2 4 dinitrochlorobenzene picryl chloride picric acid nickel salts salvarsan substituted benzoyl and benzyl chlorides acid anhydrides urushiol and so on) In both species sensitization is accomplished by repeated applications made on or into the skin and it may be necessary to provide a previously irritated cutaneous area for the treatments These technics sensitize the skin generally though in man there is some evidence for a slight delay before areas remote from the site of application acquire their sensitivity The sensitivity arises in from 5 to 20 days and is often seen first on the seventh or eighth day as a 'flare in old sites owing to a reaction with remaining traces of the sensitizing complex The full degree of sensitivity is best demonstrated by applying the same material (usually dissolved in a suitable vehicle) to a fresh area of the skin or by injecting intracutaneously either the incitant or *an artificial "conjugate antigen' made by join*ing the incitant to some soluble protein

Several results obtained by study of guinea pigs sensitized with simple chemical substances (Landsteiner Jacobs diSomma Chase Eisen) appear to apply also to the human being

The identification of sensitizing chemicals as those which can combine with proteins was *demonstrated in the case of chloro and nitro* substituted benzenes (Landsteiner and Jacobs 1936) and, conversely sensitive animals can be provoked to react by other chemically related compounds only when the latter can themselves combine with proteins (Eisen et al 1952) with most sensitizing substances however the basis of the combination with proteins remains obscure and may well reflect preliminary alteration in vivo

It has been shown that through the sensitizing procedure two different sensitivities arise in parallel—the anaphylactic type and the contact dermatitis type The balance between these is largely controlled by the chemistry of the sensitizing compound some compounds will produce chiefly a delayed type contact dermatitis others lead to both types and still others give rise largely or exclusively to the anaphylactic type

When sensitization is sufficiently intens and there is available a proper protein conjugate of the incitant systemic anaphylactic shock usually may be shown by injection of the protein conjugate into the blood stream and correspondingly the serum will lead to Passive Cutaneous Anaphylaxis when a proper test material is injected into the recipient (Chase 1947) Indeed the antibody may cir

culate undetected unless suitable protein conjugates are employed

In contrast the delayed type of dermal sensitivity not transferable with serum can be imposed upon normal guinea pigs by means of transferring washed living white cells taken from sensitized animals (Landsteiner and Chase 1942 Haxthausen 1947 Nilzen 1952) just as we have seen with the transfer of tuberculin allergy The cells not improbably lymphocytes readily confer a delayed type of sensitivity that is related specifically to the sensitivity of the donors Extracts of exudative cells have been found by Jeter Tremaine and Seebohm (1954) to effect transfer between guinea pigs Recently such cells have been reported to acquire with onset of sensitivity a special component having the mobility of alpha globulin (Jeter Lawrence and Seebohm 1957)

Unlike the transfer of tuberculin allergy it has proved to be unexpectedly difficult to transfer contact type sensitivity from man to man with cells However experimental sensitivity to 2 4 dinitrochlorobenzene has been so transferred into patients with hypogammaglobulinemia (Good et al 1957) and sensitivity to poison ivy also (Epstein and Kligman 1957) By cellular transfer *therefore* the mode of sensitivity in man appears to be established This would explain the finding of Haxthausen (1945) also made after inducing epidermal sensitivity that nonsensitive skin acquired reactivity when grafted onto a sensitized identical twin while in the reverse operation the latter s skin lost reactivity upon transplantation to its nonsensitive twin

The problem posed by the so called fixed eruptions in which sensitivity remains confined to certain fixed areas of the skin differing from one patient to another (Sulzberger 1940) would require that the skin possess a self contained factor needed for its reactivity Upon translocation by operation the skin area is said to retain its capacity to react

It is now thought that the irregularly positive results secured in attempted transfers of delayed type hypersensitivity by means of blister fluid are referable to the injection of white cells contained in the fluid The Urbach Koenigstein technic introduced originally in order to secure a special variety of cellular antibody consists in raising a blister on an area of skin that recently has undergone a specific allergic reaction and to inject the blister fluid into the skin of a normal individual subsequently the recipient is tested on the prepared site The method has been reported to transfer experimental sensitization to dinitrochlorobenzene (Ballestero and Mom 1945) Recent studies on exudates and blister fluids at the site of specific reactions have shown a surprising richness in the very types of white blood cells that are responsible for passive transfer (Nexmand 1949 1950 Baer and Yanowitz 1952 cf Fellner 1919)

When sensitization is induced with certain chemical allergens it has been found that the suspensions of cells which transfer the dermal hypersensitivity can also give rise to circulating antibody in the recipient animal (Chase 1951) apparently another demonstration of the fact that both types of sensitization represent different types of stimulus to the immunologic apparatus and can arise in parallel When donors have been stimulated with antigens in general such as bacterial vaccines and diphtheric toxoid (Harris and Harris 1951 Wager and Chase 1952) transfer of cells leads to an appearance of antibody but does not lead to production of delayed type sensitivity

The mechanism underlying drug sensitization appears to be involved intimately with the antibody producing system which itself is not understood as yet That one is dealing in the establishment of delayed type sensitivities with some part of the antibody producing mechanism seems also to follow from some other observations First dermal sensitivity of the delayed type can be induced by an insoluble complex antigen (made of the chemical coupled to erythrocyte stromata) when it is used along with the Freund adjuvant (Landsteiner and Chase 1941) Secondly when one feeds certain allergenic chemicals to nonsensitized animals there is induced a state of specifically depressed receptivity involving both the acquisition of delayed type *dermal sensitivity* and the production of antibody The animal acquires resistance to a subsequent dermal sensitization with this chemical and upon stimulation with conjugated homologous antigens bearing the same immunologically determinant structure it fails to become anaphylactic or to exhibit corresponding circulating antibodies (Chase 1946 1949 Battisto and Chase 1955a b) The phenomenon *may well* depend upon a retention of the determinant structural grouping in the tissues

in such wise as to modify the antibody producing apparatus

In man immediate type, flare and wheal reactions to drugs have been found in several instances (salvarsan, formaldehyde phthalic anhydride chloramine T sulfathiazole and sulfadiazine) and are often accompanied by demonstrable reagins it is evident that these incitants which are capable of giving early type reactions would largely belong in the category of the more reactive compounds It may well be that as in the case of sensitized guinea pigs these reagins may occur more commonly than has been recognized, and that their detection may wait upon the use of suitable protein conjugates

In order to search for antibodies that may be present even in low concentrations Coombs Mynors and Weber (1950) and Coombs and Fiset (1954) have suggested novel methods for preparing test antigens The erythrocytes would be coated with a specifically combining but nonagglutinating antibody (using for example human red cells and the human incomplete Rh antibody or various rabbit antibodies that have been rendered nonagglutinating by photo oxidation) to which the desired allergen had been linked chemically This device would permit use of the sensitive reaction of agglutination the erythrocytes bearing the desired allergenic groups being tested versus serum suspected of containing antibody directed against the allergen in question Even amounts of antibody too minute to cause agglutination should combine with the complex and theoretically be detectable by applying indirect tests Indeed their method should be useful in instances in which a coupling to protein is possible without enslaving the immunologically determinant sites of the allergens

Finally it may be said that attempts at desensitization in contact dermatitis and in drug allergy have met with limited success some cases recede spontaneously a few others have been benefited by subcutaneous injection of the incitant in an oil vehicle (Caulfield 1936) or by cautious ingestion (Park 1944 Stevens 1945) A useful method has not yet been evolved and except for spontaneous recession the sensitivity soon returns as is true in all other types of allergic manifestations

For use in special studies it may be mentioned that guinea pigs can be *prevented* from undergoing specific sensitization to certain simple chemicals by certain elective experiences induced with the chemical before attempting experimental sensitization (intravenous injection of arsphenamine Sulzberger feeding of fat soluble allergenic chemicals, Chase)

Theoretical Considerations of Delayed Type Hypersensitivity

As we have seen, bacterial infection leads characteristically, but not exclusively to a delayed type of reactivity whereas various extracts of the same bacterial cells injected separately give rise only to the anaphylactic type of sensitivity It is probable that the latter sort of stimulus occurs always, even if inappreciably along with the more obvious development of the delayed type of reactivity In the case of tubercle bacilli Raffel has pointed to the presence of a special waxy material said to be antigenically inert that possesses a remarkable 'directive effect in inducing the production of delayed type hypersensitivity toward admixed tuberculoprotein or other proteins (White in Shaffer et al 1958) The "directive effect may well be the attraction of cellular types such as monocytes and cells of the lymphatic series that result in the local granulomatous response which the mycobacteria induce In addition as White Coons and Connolly (1955) point out the increased amount of antibody observed when mycobacterial wax is used is accompanied by a widespread proliferation of plasma cell elements Perhaps analogous directing substances may occur in diminutive (but effective) amounts in other microbial forms As Swift and Derick (1929) have stated In practically all instances in which a tuberculin like allergy is induced this follows some focal tissue reaction resulting from injury by the respective microorganisms

However it is obvious that a broadened base must be sought owing to the very multiplicity of agents that are capable of inducing delayed type sensitivity Among these there are included drugs and poison ivy which function as allergens after direct application on or into the skin and by combination with tissues perhaps may lead to cellular responses that are equivalent to focal reactions

For the special case of chemical allergens Mayer (1957) has suggested that fibroid proteins such as collagen, rather than soluble proteins may be the necessary carrier for the coupling that leads to delayed type hypersensitivity However radio labeled allergens appear to escape from the skin rather readily (Eisen Ritts) Mayer's evidence rests upon his finding that procollagen may be substituted for mycobacteria or mycobacterial wax in a special procedure of sensitizing the skin by injecting a chemical allergen intraperitoneally (cf Landsteiner and Chase 1939) The conclusion that the usual role of mycobacteria is to allow local accumulation of collagen as in the peritoneal cavity must be subjected to careful scrutiny

The intracutaneous route of injection is outstandingly successful in leading to sensitivities of the delayed type and is not confined to use of chemical allergens As Dienes found in 1929 the direct intracutaneous injection of native proteins leads to a delayed type of reactivity as the first response of the guinea pig recent experiments by Salvin (1958) show that if the intracutaneous dose be small (e g 3 μg) the delayed type of response may be maintained for several weeks before antibody appears in the circulation and Arthus type reactivity becomes manifest The small size of the dose may be important in determining how readily the injected material escapes via the lymphatics and contributes to widespread antigenic stimuli Similarly when antigen is masked by an overcoating with specific antibody (the complex being formed in the region of antibody excess) the period during which the delayed type of response is present may be very much prolonged (Uhr Salvin and Pappenheimer 1957 Salvin 1958) The latter technic has even been employed to establish delayed type sensitivity in man to diphtheric toxoid essentially without production of antitoxin and it has been shown that the resultant delayed type sensitivity can be transferred to other individuals by means of white cells (Good et al 1957) Such results as well as the finding of delayed type allergies in hypogammaglobulinemic individuals in whom antibody production is not to be stimulated have pointed to delayed type hypersensitivity as a separate and real entity that calls for separate explanations from ordinary antibody production

Gell and Hinde (1954) have suggested that delayed type hypersensitivity results from an antigenic stimulus that stops short of inducing plasma cell formation being confined to mononuclear cells In extension of this thought Pappenheimer (cf Shaffer et al 1958) proposes that delayed type hypersensitivity may occur always as a first step in the production of antibody and represents a mechanism analogous to induced enzyme formation that prepares the altered cells to produce circulating antibody electively upon a subsequent experience with the same antigen The necessary corollaries that delayed type hypersensitivity always precedes antibody formation and that it becomes superseded when circulating antibody appears are not sustained by all available facts

The hypothesis of cell bound antibody has been offered to explain the injury done by tuberculin to monocytes of the tuberculous host and by derivation the phenomenon of delayed type hypersensitivity in general Burnet for example once suggested that eventually there is produced and put into the blood stream a special antibody of such variety that it binds to the tissues with extreme readiness and is seldom to be encountered in the blood in amount sufficient to permit passive transfer The concept and previously mentioned evidence of a specific transfer factor i the closest that one has yet approached to the idea of cell bound antibody and lymphocytes rather than monocytes have been regarded as the prototypic cell Even in imposing delayed type hypersensitivity by means of cellular transfer it does not seem to be impossible that the transferred cells give rise to an active participation of host cells even when they are not subjected to lysis prior to transfer Evidently we are on the verge of understanding a long mysterious phenomenon

Homograft Rejection

Rejection of tissue homografts when these are made between normal individuals of a species occurs regularly unless genetic homozygosity exists between donor and recipient (identical twins highly inbred *strains of* mice and guinea pigs) The manner of rejection and the speedier rejection of a later identical graft

point to an immunologic mechanism Similarities between homograft rejection and tuberculin type allergy have been outlined by Lawrence (1957) *Consonant with this interpretation*, it has been shown that tissue can continue to grow within a noncompatible host provided that it is isolated from the host's cells by a cell impermeable membrane (Algire et al 1954 1957) However others are inclined to see a role for circulating antibody in homograft rejection (cf Voisin and Maurer 1957 Stetson 1958)

The reverse situation, homograft tolerance is presently under active study because of its theoretical implications The artifice of establishing tolerance through prenatal experience with cellular antigens (white cells of spleen peripheral blood lymph nodes) was established by the brilliant experiments of Medawar Billingham and Brent the subject has been reviewed *in extenso* by Brent (1958)

THE SHWARTZMAN PHENOMENON

There remains to be mentioned a special sort of hemorrhagic necrotic reaction that can be produced in the skin and some other organs (chiefly the kidney) but it is not dependent upon an antigen antibody mechanism nonetheless its participation in acute disease processes is frankly suspected As will be obvious it possesses features in common with the Arthus reaction We refer to the phenomenon of local tissue reactivity developed by Shwartzman[6] and described also under the name of the Sanarelli Shwartzman phenomenon In the original experiment certain bacteria or culture filtrates of these were injected into the skin of rabbits and after a lapse of from 8 to 32 hours a filtrate from the same culture was introduced in quantity into the blood stream within a few hours gross hemorrhage and necrosis developed at the prepared sites It was found however that the skin preparatory factors and the reacting factors need not be identical or even immunologically related For example the local preparatory injection may be made with culture filtrates of *E coli* or *S typhosa* and the intravenous eliciting injection with meningococcal culture filtrates Only certain bacterial strains will serve to provide adequate yields of Shwartzman toxin for the local preparation or "sensitization of the skin some strains are said to afford only the preparatory factor, others chiefly the reacting factor

For the local nonspecific sensitization of the skin, simple inflammatory agents do not replace the use of selected bacterial filtrates The active material in the culture filtrate is usually identified with bacterial protein—reputedly with the endotoxins or complete somatic antigens—and is antigenic, however a nitrogen free polysaccharide from *S marcescens* will both prepare and elicit the reaction (Black Schaffer et al, 1950) The rabbit is the animal of choice for this experiment goats and horses show the effect guinea pigs respond irregularly and less intensely and mice and rats were not found to be susceptible to skin preparation In special strains of mice, however, Shwartzmanlike reactions have been noted to an initial injection of bacterial polysaccharide (Kelly et al 1957)

While both of the injections were originally made with culture filtrates of selected bacteria either of the two may be replaced in various ways It is possible to prepare the skin site *with a bacterial culture filtrate and to elicit* the reaction by an intravenous injection of certain nonbacterial materials—starch glycogen agar, or washed antigen antibody precipitates all of which incidentally alter guinea pig serum in vitro so that it gives rise to anaphylactoid shock Or if a skin site is prepared with a culture filtrate in an immunized rabbit intravenous injection of the corresponding antigen will result by means of an interaction between antigen and antibody functioning as the eliciting factor in a typical necrotic lesion of Shwartzman type this is not surprising apart from the special qualities of the lesion that are a characteristic of the preparatory toxin and the delicacy of the reaction since chilling or irritation of the skin under similar operating conditions can cause local allergic inflammation The reverse situation namely substituting for the preparatory injection a fully developed tuberculin reaction on a tuberculous animal and giving later an intravenous injection of a potent Shwartzman filtrate or even tuberculin also results in a typical Shwartzman reaction (Freund 1934 guinea pig Stetson 1955 rabbits) tumor tissue in mice offers a naturally prepared situs for Shwartzman effects and responds with inflammation when potent bacterial filtrates

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are injected intravenously and sometimes magnification of the Arthus reaction in its early stages can be secured by injecting into the skin of an immunized animal in mixture with the specific antigen a Shwartzman reacting factor (Black Schaffer et al 1950) in these cases the antigen antibody reaction appears to substitute for the preparatory factor

An explanation of the Shwartzman effect has been long sought From studies chiefly by Thomas and Stetson (1949) and Stetson (1951) a partial picture can be envisioned As a consequence of the preparatory injection polymorphonuclear leukocytes accumulate locally in the tissue around the smaller veins as cuffs and probably chiefly owing to the metabolic character of exudate polymorphonuclear leukocytes abnormal quantities of lactic acid are produced there through aerobic glycolysis Changes (histologically inapparent) then occur in the adjacent vascular endothelium perhaps of a sort that render this tissue sensitive to the action of tissue protease Then upon the intravenous eliciting injection a peripheral vasoconstriction occurs impairing the blood supply white cells and blood platelets clump together forming masses that are removed from the circulation and become sequestered in the lung perhaps the liver and the spleen and markedly along the damaged endothelium In the latter areas the aggregates form leukocyte platelet thrombi and actually occlude the small vein and the capillaries The death and the disintegration of involved vessels and of cells in the inflammatory exudate occurs within the following 2 or 3 hours with activated tissue proteases perhaps playing a role along with interruption of the blood supply The characteristic and prominent hemorrhage in the prepared site would follow as a consequence of necrosis of the vessel walls

As Rall et al (1957) *point out adrenergic* vasoconstriction accompanying the eliciting injection could enhance local damage since epinephrine is known to exaggerate Shwartzman effects

Several features recall events encountered in the allergic state First there is the early and profound leukopenia noted in (nonexplosive) anaphylactic shock of the guinea pig the dog and the rabbit and in the Arthus reaction together with sequestration of cells and platelets in lung capillaries and other sites of the vascular endothelium Then one can desensitize by repeated small doses of the eliciting material (Cluff and Bennett 1951) Again the accumulation of lactic acid in the site of reaction as well as the packing of the platelet leukocyte thrombi is alike in the Shwartzman and the Arthus reaction (Stetson 1951b) and finally in both of these instances the gross development of local tissue damage is similar After making parallel studies on tuberculin sensitive rabbits injected with Old Tuberculin Stetson (1955) has even suggested that the biologic activity of endotoxins may reflect a delayed type natural hypersensitivity of the rabbit to gram negative bacteria namely fever responses to pyrogen and tuberculin respectively short term ear blanching intracorneal damage and direct delayed type toxic injury are much alike and as stated above tuberculin can give Shwartzman effects in tuberculin sensitized animal However the parallelism is insufficiently complete to indicate cause and effect Perhaps one could say that the Shwartzman event is to the Arthus and the delayed type allergies what the anaphylactoid is to the anaphylactic shock namely parallel and interwoven mechanism for obtaining the same ultimate tissue response

Another aspect is the *generalized Shwartzman* reaction in which both injections must be given intravenously characteristically starting from 6 to 8 hours after the eliciting injection there is deposition of a fibrinoidlike homogeneous material particularly within the glomerular capillaries of the kidney which leads to occlusion of the circulation and results in bilateral renal cortical necrosis In one sense this mechanism contrasts sharply with the *local* Shwartzman reaction with its *cellular* occlusion of local blood vessels failure to deposit amorphous material and absence of renal necrosis nevertheless neither the local nor the generalized Swartzman phenomena occurs in nitrogen mustard induced leukopenic states Special effects have been *noted in the generalized Shwartzman reaction* for example when certain anticoagulant materials are combined with the preparatory injection of endotoxin there is a profound drop in fibrinogen and death ensues with typical findings in the kidneys without requirement for a second intravenous injection

There has been active speculation that natural infections with bacteria may cause tissue preparation and that Shwartzman effects can occur owing to reacting factors provided by the infection itself by a proximal antigen antibody reaction or by a variety of other nonspecific stimuli this has been surmised to

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An explanation of the Shwartzman effect has been long sought From studies chiefly by Thomas and Stetson (1949) and Stetson (1951), a partial picture can be envisioned As a consequence of the preparatory injection polymorphonuclear leukocytes accumulate locally in the tissue around the smaller veins as cuffs and probably chiefly owing to the metabolic character of exudate polymorpho nuclear leukocytes abnormal quantities of lactic acid are produced there through aerobic glycolysis Changes (histologically inappar ent) then occur in the adjacent vascular endo thelium perhaps of a sort that render this tissue sensitive to the action of tissue protease Then upon the intravenous eliciting injection a peripheral vasoconstriction occurs impair ing the blood supply white cells and blood platelets clump together forming masses that are removed from the circulation and become sequestered in the lung perhaps the liver and the pleen and markedly along the damaged endothelium In the latter areas the aggre gates form leukocyte platelet thrombi and actually occlude the small veins and the capil laries The death and the disintegration of involved vessels and of cells in the inflamma tory exudate occurs within the following 2 or 3 hours with activated tissue proteases per haps playing a role along with interruption of the blood supply The characteristic and prominent hemorrhage in the prepared site would follow as a consequence of necrosis of the vessel walls

As Rall et al (1957) point out adrenergic vasoconstriction accompanying the eliciting in jection could enhance local damage since epi nephrine is known to exaggerate Shwartzman effects

Several features recall events encountered in the allergic state First there is the early and profound leukopenia noted in (nonexplo sive) anaphylactic shock of the guinea pig the dog and the rabbit and in the Arthus reaction together with sequestration of cells and platelets in lung capillaries and other sites of the vascular endothelium Then one can desensitize by repeated small doses of the eliciting material (Cluff and Bennett 1951) Again the accumulation of lactic acid in the site of reaction as well as the packing of the platelet leukocyte thrombi is alike in the Shwartzman and the Arthus reaction (Stetson 1951b) and finally in both of the e instances the gross development of local tissue damage is similar After making parallel studies on tuberculin sensitive rabbits injected with Old Tuberculin Stetson (1955) has even suggested that the biologic activity of endotoxins may reflect a delayed type natural hypersensitiv ity of the rabbit to gram negative bacteria namely fever responses to pyrogen and tu berculin respectively short term ear blanch ing intracorneal damage and direct delayed type toxic injury are much alike and as stated above tuberculin can give Shwartzman effects in tuberculin sensitized animals How ever the parallelism is insufficiently complete to indicate cause and effect Perhaps one could say that the Shwartzman event is to the Arthus and the delayed type allergies what the anaphylactoid is to the anaphylactic shock namely parallel and interwoven mechanisms for obtaining the same ultimate tissue re sponse

Another aspect is the *generali ed Shwart man* reaction in which both injections must be given intravenously characteristically starting from 6 to 8 hours after the eliciting injection there is deposition of a fibrinoidlike homogeneous material particularly within the glomerular capillaries of the kidney which leads to occlusion of the circulation and re sults in bilateral renal cortical necrosis In one sen e this mechanism contrasts sharply with the *local* Shwartzman reaction with its *cellular* occlusion of local blood vessels fail ure to deposit amorphous material and ab sence of renal necrosis nevertheless neither the local nor the generalized Swartzman phe nomena occurs in nitrogen mustard induced leukopenic states Special effects have been noted in the generalized Shwartzman reaction for example when certain anticoagulant ma terials are combined with the preparatory in jection of endotoxin there is a profound drop in fibrinogen and death ensues with typical findings in the kidneys without requirement for a second intravenous in ection

There has been active speculation that natu ral infections with bacteria may cause tissue preparation and that Shwartzman effects can occur owing to reacting factors provided by the infection itself by a proximal antigen antibody reaction or by a variety of other nonspecific stimuli this has b en urmised to

point to an immunologic mechanism Similarities between homograft rejection and tuberculin type allergy have been outlined by Lawrence (1957) Consonant with this interpretation it has been shown that tissue can continue to grow within a noncompatible host provided that it is isolated from the host's cells by a cell impermeable membrane (Algire et al 1954, 1957) However others are inclined to see a role for circulating antibody in homograft rejection (cf Voisin and Maurer, 1957 Stetson, 1958)

The reverse situation homograft tolerance is presently under active study because of its theoretical implications The artifice of establishing tolerance through prenatal experience with cellular antigens (white cells of spleen peripheral blood lymph nodes) was established by the brilliant experiments of Medawar Billingham and Brent the subject has been reviewed *in extenso* by Brent (1958)

THE SHWARTZMAN PHENOMENON

There remains to be mentioned a special sort of hemorrhagic necrotic reaction that can be produced in the skin and some other organs (chiefly the kidney) but it is not dependent upon an antigen antibody mechanism nonetheless its participation in acute disease processes is frankly suspected As will be obvious it possesses features in common with the Arthus reaction We refer to the phenomenon of local tissue reactivity developed by Shwartzman[6] and described also under the name of the Sanarelli Shwartzman phenomenon In the original experiment certain bacteria or culture filtrates of these were injected into the skin of rabbits and after a lapse of from 8 to 32 hours a filtrate from the same culture was introduced in quantity into the blood stream within a few hours gross hemorrhage and necrosis developed at the prepared sites It was found however that the skin preparatory factors and the reacting factors need not be identical or even immunologically related For example the local preparatory injection may be made with culture filtrates of *E coli* or *S typhosa* and the intravenous eliciting injection with meningococcal culture filtrates Only certain bacterial strains will serve to provide adequate yields of Shwartzman toxin' for the local preparation or "sensitization' of the skin some strains are said to afford only the preparatory factor others chiefly the reacting factor

[6] The reader is referred to Shwartzman (1937) to Wilson and Miles (1955) and to literature cited

For the local, nonspecific sensitization of the skin, simple inflammatory agents do not replace the use of selected bacterial filtrates The active material in the culture filtrate is usually identified with bacterial protein—reputedly with the endotoxins or complete somatic antigens—and is antigenic however, a nitrogen free polysaccharide from *S marcescens* will both prepare and elicit the reaction (Black Schaffer et al, 1950) The rabbit is the animal of choice for this experiment goats and horses show the effect guinea pigs respond irregularly and less intensely and mice and rats were not found to be susceptible to skin preparation In special strains of mice, however Shwartzmanlike reactions have been noted to an initial injection of bacterial polysaccharide (Kelly et al 1957)

While both of the injections were originally made with culture filtrates of selected bacteria either of the two may be replaced in various ways It is possible to prepare the skin site with a bacterial culture filtrate and to elicit the reaction by an intravenous injection of certain nonbacterial materials—starch glycogen agar or washed antigen antibody precipitates all of which incidentally alter guinea pig serum in vitro so that it gives rise to anaphylactoid shock Or if a skin site is prepared with a culture filtrate in an immunized rabbit, intravenous injection of the corresponding antigen will result by means of an interaction between antigen and antibody functioning as the eliciting factor in a typical necrotic lesion of Shwartzman type this is not surprising apart from the special qualities of the lesion that are a characteristic of the preparatory toxin and the delicacy of the reaction since chilling or irritation of the skin under similar operating conditions can cause local allergic inflammation The reverse situation namely substituting for the preparatory injection a fully developed tuberculin reaction on a tuberculous animal and giving later an intravenous injection of a potent Shwartzman filtrate or even tuberculin also results in a typical Shwartzman reaction (Freund 1934 guinea pig Stetson 1955 rabbits) tumor tissue in mice offers a 'naturally prepared situs for Shwartzman effects and responds with inflammation when potent bacterial filtrates

ACTH and Cortisone

Following the introduction of porcine ACTH (pituitary adrenocorticotropic hormone) in 1946 many diverse disease processes were found to be markedly ameliorated and among these were nearly all varieties of allergic manifestations Shortly afterward compound E of the adrenal cortex (cortisone) became available and was found to influence markedly the allergic state Recently other compounds including partially synthesized materials have been developed (cf Jailer 1955)

Cortisone and hydrocortisone as Germuth (1956) has remarked possess a marked anti inflammatory effect owing perhaps to a vaso constrictive action that leads to reduction in blood flow and inhibition of capillary permeability there is increased vascular tone lessened diapedesis and inhibition of lymphocytic reproduction These materials prove to be effective in relieving intrinsic and extrinsic asthma they control allergic eczema as well Likewise they are of benefit in the collagen vascular diseases

Stringent medical supervision is required The physician must weigh carefully the temporary benefits that the patient may derive against the grave disadvantages that come from exhaustion of the adrenals and impairment of the normal hypothalamus pituitary adrenal axis Since inflammatory responses are suppressed there is the hazard with continued use that serious bacterial infection may become established With ACTH there is occasional allergization which is usually pecific for the pecies of origin A hormone fastness has also been encountered infrequently

Although initial symptomatology usually returns with cessation of treatment in instances in which the offending allergen can be expected to disappear within a definitely limited time from environment (pollens) or from tissue sites (e g following a reaction to injected penicillin) there may be no further symptoms after treatment is terminated

Attempts to account for the observed measure of clinical improvement solely as a parallel reduction in concentration of circulating antibody have failed even though production of antibody appears largely to cease upon hormonal treatment (Bjørneboe Fischel and Stoerk 1951 Germuth Oyama and Ottinger 1951) Rose et al (1950) subjected patients under treatment with ACTH to histamine and mecholyl and demonstrated actual resistance to dyspnea and bronchoconstriction In experimental systemic anaphylaxis with actively sensitized animals treated dogs and mice exhibited diminution in their responses but not treated guinea pigs this difference may well be one of degree possibly reflecting only the intensity of the guinea pig s re ponse to antigen Treatment with these hormones does not affect the interaction of antigen and antibody Passive Arthus reactions can be evoked typically (Germuth and Ottinger 1950), and Carryer and Code (1950) showed that the presence of cortisone did not hinder the release of histamine by rabbit blood under the stimulus of a hemolytic antigen antibody reaction The Shwartzman reaction in rabbits is inhibited by use of the hormone and even the development of experimental periarteritis nodosa in rabbits is largely hindered by use of ACTH

By intensive treatment of animals with the hormones delayed type reactions as to tuberculin or streptococcal vaccine are diminished but in varying degree Such suppressive effects have been observed in sensitized rabbits (Harris and Harris 1950 Gell and Hinde 1951) and in guinea pigs (Long and Miles 1950 Osgood and Favour 1951) A prototype has been seen in the relative inertness of intensively treated animals in giving inflammatory responses upon injection of oil of turpentine (Osgood and Favour 1951 cf Gell and Hinde 1951) The suggestion has been made that the metabolism of ascorbic acid (Long 1952) may be involved in the failure of such treated animals to react to tuberculin

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* Recent books that present pecial aspects of allergy with bibliography are Wilson and Miles (1955) Lawrence (1958) and Shaffer LoGrippo and Chase (1958)

be one possible factor operating in bacterial allergy and in the genesis of conditions such as pulmonary abscess or hemorrhagic lesions seen in meningococcemia (Black Shaffer et al 1947) However, no direct proof is at hand It is suggestive that extracts of streptococcal lesions in rabbit skin contain a substance functioning as preparatory factor (but not extracts of the streptococci themselves) and that streptolysin filtrates in the reduced state serve to evoke the reaction at these sites (Schwab Watson and Cromartie 1953)

MODIFICATIONS IN THE ALLERGIC STATE

Certain physiologically active materials serve temporarily to mitigate or suppress various of the allergic manifestations Both caution and experience are required in clinical application *There are the so called antihistamines* pituitary adrenocorticotrophic hormone (ACTH) cortisone (compound E) hydrocortisone fluorohydrocortisone prednisone prednisolone and still others

Antihistamines

The development of the histamine theory emphasizing the role of histamine in anaphylactic shock and in allergic reactions of *immediate type excited the hope that the* physiologic consequences of antigen antibody interaction might be avoided if the liberated histamine could be rendered ineffective Various approaches have been explored such as *administration of the enzyme histaminase* immunization by an artificial antigen' containing histamine coupled to a protein and more recently administration of special compounds known to have antihistaminic properties This last approach opened unexpectedly by work of Bovet and Staub in 1937 has provided a powerful adjunct but not a panacea in treatment of allergic conditions The compounds influence in some degree other physiologically active substances as well (Loew 1950 Feinberg et al 1950) and are effective only so long as they can be maintained in histamine sensitive tissues at an adequate level with respect to the molecular concentration of histamine They are presumed to function as 'competitive inhibitors for the sites affected by histamine

The first effective compound introduced in 1942, was *Antergan* (N benzyl N', N dimethyl N phenyl ethylenediamine) after which a multitude of other structures were synethesized (see Feinberg et al 1950) As with other proprietary compounds the products possess not only their trade names but also type' names thus Pyribenzamine is one brand of 'Tripelennamine hydrochloride Histadyl and Thenylene are different brands of '*Methapyrilene hydrochloride Benadryl* is a brand of Diphenhydramine hydrochloride and so on

Use of these substances by allergic individuals should be under medical supervision The *variety in formulae has been welcomed be*cause of individual reactions and requirements All these compounds can produce undesirable 'side reactions as drowsiness dizziness disorientation and gastro intestinal disturbances *occasionally (with overdose) even death*

These compounds often prove to be valuable in cases of allergic urticaria and allergic rhinitis and they diminish the itching and certain other manifestations accompanying urticaria *angioneurotic edema and serum disease* though asthma especially is apt to be resistant Since the compounds function also as local anesthetics somewhat more so in this respect than procaine they may relieve the itching in *poison ivy dermatitis but they do not modify* in fundamental manner reactions that are of the delayed type

The evidence for the protective action of *these compounds against the effects produced* in a normal animal by administration of histamine is impressive it extends to protection of the isolated guinea pig gut or uterine horn from contraction by histamine and to inhibition of vascular effects of histamine in the rabbit and other animals to the protection of guinea pigs from multiple lethal doses of histamine and from the bronchospasm caused by histamine aerosol At the same time it is found that most of these compounds do not *modify the gastric secretogogue action of* histamine

When the antihistaminic compounds are tested for ability to protect anaphylactically sensitized guinea pigs against systemic shock by an intravenous injection of antigen larger amounts are necessary (3 mg/Kg down to 1 mg/Kg) than are needed to deviate the effects of purely histamine shock

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Human Blood Groups

INTRODUCTION

In 1900 Landsteiner noted the clumping or agglutination of normal human red blood cells when mixed with the serum of certain other normal individuals This agglutination was traced to the variable distribution of 2 antigens or blood factors in the red blood cells and their corresponding antibodies i e iso agglutinins * These observations led to the discovery of the 4 blood groups and the recognition of their importance in transfusions Thus an explanation became available for the early transfusion accidents in man and a basis was provided for the proper selection of compatible blood i e the recipient and the donor must belong to the same blood group (Landsteiner 1931)

Numerous other blood factors have since been recognized in human red blood cells such as M N P Rh Hr K k S s etc With rare exceptions there are no naturally occurring iso agglutinins for these blood factors accordingly they present no problem in the primary blood transfusion However iso agglutinins may arise as a result of transfusions of blood containing factors foreign to the recipient and as a result of pregnancy where the fetus inherits from the paternal line blood factors not present in the mother This phenomenon of iso immunization by pregnancy makes it necessary to invoke additional criteria of compatibility between recipient and donor

It required an interval of 40 years before iso immunization in man was established as the cause of intragroup hemolytic transfusion reactions in recipients of repeated transfusions or in recently pregnant women at the very first transfusion In 1940 41 it was shown that iso immunization of the mother by fetal blood and the subsequent reaction in utero of maternal antibodies with susceptible fetal blood was the cause of the condition known as hemolytic disease of the fetus and the newborn In the latter instance and in intragroup transfusion reactions the most important blood factor involved is the agglutinogen called Rh its corresponding immune iso antibody is anti Rh or anti D to be described below Iso immunization by the Rh factor is responsible for the vast majority of all cases of intra group hemolytic disease of the newborn and intragroup hemolytic transfusion reactions

* Ant bod es prepared against ant gens of animal origin have been given the prefix hetero when produced by a species other than that furnishing the antigen The e may be designated as immune hetero antibodies in contrast with normal heteroantibodies which are frequently present in normal animal serum Isoantibodies which differentiate indiv duals within the same species are also either normal or immune in origin As examples of normal isoantibodies are those which define the 4 blood groups Immune isoa tibod s are produced by individuals in re po e to antigenic differences within the same speci The phenomenon of isoimmunization was demon trated for the first t me i 1900 by Ehrlich d Mo g nroth in experime ts with goats

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Human Blood Groups

INTRODUCTION

In 1900 Landsteiner noted the clumping or agglutination of normal human red blood cells when mixed with the serum of certain other normal individuals This agglutination was traced to the variable distribution of 2 antigens or blood factors in the red blood cells and their corresponding antibodies i e isoagglutinins * These observations led to the discovery of the 4 blood groups and the recognition of their importance in transfusions Thus an explanation became available for the early transfusion accidents in man and a basis was provided for the proper selection of compatible blood i e the recipient and the donor must belong to the same blood group (Landsteiner 1931)

Numerous other blood factors have since been recognized in human red blood cells such as M N P Rh Hr K k S s etc With rare exceptions there are no naturally occurring iso-agglutinins for these blood factors accordingly they present no problem in the primary blood transfusion However isoagglutinins may arise as a result of transfusions of blood containing factors foreign to the recipient and as a result of pregnancy where the fetus inherits from the paternal line blood factors not present in the mother This phenomenon of iso-immunization by pregnancy makes it necessary to invoke additional criteria of compatibility between recipient and donor

It required an interval of 40 years before iso-immunization in man was established as the cause of intragroup hemolytic transfusion reactions in recipients of repeated transfusions or in recently pregnant women at the very first transfusion In 1940–41 it was shown that iso-immunization of the mother by fetal blood and the subsequent reaction in utero of maternal antibodies with susceptible fetal blood was the cause of the condition known as hemolytic disease of the fetus and the newborn In the latter instance and in intragroup transfusion reactions the most important blood factor involved is the agglutinogen called Rh its corresponding immune iso-antibody is anti Rh_0 or anti D to be described below Iso-immunization by the Rh factor is responsible for the vast majority of all cases of intra group hemolytic disease of the newborn and intragroup hemolytic transfusion reactions

* Antibodies prepared against antigens of animal origin have been given the prefix hetero when produced by a species other than that furnishing the antigen These may be designated as immune hetero antibodies in contrast with "normal heteroantibodies which are frequently present in normal animal serum Isoantibodies which differentiate individuals within the same species are also either normal or immune in origin As examples of normal isoantibodies" are those which define the 4 blood groups Immune isoantibodies are produced by individuals in response to antigenic differences within the same species The phenomenon of isoimmunization was demonstrated for the first time in 1900 by Ehrlich and Morgenroth in experiments with goats

The serologic analysis of the few remaining cases not attributable to the Rh factor revealed the presence of numerous new blood factors Still other blood factors have been described with the aid of naturally occurring and therefore normal iso agglutinins so that at long last the concept of the individuality of human red blood cells is now firmly established

THE 4 BLOOD GROUPS

On testing normal human sera with red cells of numerous individuals 4 types of reactions are observed as shown in Table 16

TABLE 16 THE 4 BLOOD GROUPS
Properties of the Red Blood Cells and the Serum

BLOOD GROUP	ANTIBODIES PRESENT IN THE SERUM	REACTION OF SERUM AND RED BLOOD CELLS OF GROUP			
		O	A	B	AB
O	Anti A Anti B	o	+	+	+
A	Anti B	o	o	+	+
B	Anti A	o	+	o	+
AB	None	o	o	o	o
Incidence (%) in white population of U S A		45	41	10	4

Landsteiner had assumed the existence of 2 agglutinable substances A and B and their corresponding iso agglutinins anti A and anti B the distribution of which is responsible for the classic 4 blood groups Groups O and AB are readily distinguished by the absence of A and B factors in group O blood and their combined presence in group AB Their sera are characterized by the presence of both anti A and anti B in group O individuals and the absence of these iso antibodies in group AB individuals

The 4 blood groups can be determined by simple agglutination tests with 2 reagents i e the serum of group A individuals as a source of anti B alone and the serum of group B individuals containing anti A alone These relationships are indicated in Table 16

Bloods are compatible if the patient's serum does not agglutinate the donor's red blood cells This condition of major compatibility is satisfied if patient and donor belong to the same blood group and if intragroup incompatibility, mainly due to the Rh factor is excluded

Because group O bloods lack the factors A and B, their red blood cells are compatible with the 3 other groups so that they have been designated as 'universal donors" By the same token, group AB individuals are universal recipients, since their serum contains neither anti A nor anti B and they can receive transfusions from individuals of groups O A or B

In the use of group O individuals as universal donors there is a minor incompatibility resulting from the presence of injected iso agglutinins capable of reacting with the recipient s red cells In the vast majority of the cases there is a wide margin of safety since the agglutinins in one unit of blood (500 cc) are as a rule sufficiently diluted and/or neutralized in the recipient s circulation However these quantitative relationships may be upset if the titer of anti A or anti B of the universal donor s blood is unusually high as a result of antigenic stimuli due to plasma transfusions pregnancies with group A or group B offspring or antigenic stimuli of uncertain origin In administering such blood particularly to anemic patients with a reduced blood volume there may be an insufficient dilution of the transfused iso agglutinins which may then destroy the patient s red blood cells Because of some special properties of anti A and anti B of group O sera (higher titers and cross reactivity referred to below) these considerations are not as applicable to the anti B of group A donors or anti A of group B donors for group AB recipients

The simple scheme shown in Table 16 applies to the vast majority of all bloods but it does not take into account weaker forms of groups A and more rarely weaker forms of group B nor the exceptions and the variations in qualitative or quantitative content of anti A and anti B

BLOOD GROUP SUBSTANCES

The iso agglutinogens A and B are as a rule already present at birth They are constitutional hereditary properties which are constant throughout life The A and B properties are present not only in the red blood cells but have been demonstrated in other tissue cells such as spermatozoa liver and epi

thelial cells The great stability of these substances permits the determination of the blood group of mummified tissue or blood dried for many years Group specific substances are found also in body fluids (saliva seminal fluid urine and serum) of most individuals who are known as secretors in the smaller group known as nonsecretors the A and B substances are not present in body fluids Group O individuals are also classified as secretors or nonsecretors of a basic substance called H The secretor status is inherited as a mendelian dominant while the nonsecretor status is recessive In both secretors and nonsecretors alcohol soluble group specific substances can be extracted from organs as well as red blood cells

A factor related to the group A substance is widely distributed in nature and has been demonstrated in the Forssman antigen of certain bacteria and of gastric mucosa of pig and horse Factors related to A B and H have been shown to be present in the gastric mucosa of individual horses Because they are readily available pig and horse gastric mucosa have been employed for the chemical preparation of group A B and H (O) substances They are all polysaccharide amino acid complexes and most recently A B and H (O) substances whether prepared from animal sources or human sources have been shown to have specific terminal groupings (1 fucose and derivatives for H or O β N acetyl D galactosamine and derivatives for A and α D galactose and derivatives for B)

The group specific A and B substances have commonly been used for the operation of blood banks because of their capacity to diminish or neutralize the iso agglutinin content of selected universal donor blood Furthermore their antigenicity in man makes it possible to obtain high titered serum for rapid and accurate blood group determination The group specific substances are also useful in the processing of specific diagnostic anti Rh and other *isoimmune sera*

The Subgroups A_1 and A

All bloods of groups A and AB can be subdivided into two broad classes called A_1 (A_1B) and A (A B) In tests with freshly drawn group O or B sera about 80 per cent of the bloods A_1 will be hemolyzed while the remainder A which are more or less resistant to hemolysis will be clumped Subgroups A_1 and A of groups A and AB can also be selected on the basis of intensity of agglutination in tests with inactivated sera (56 C for 15 minutes) A_1 being more sensitive than A

The same differentiation may be made with the aid of rare naturally occurring atypical iso agglutinins anti A_1 and anti A Anti A is now more commonly referred to as anti H or anti O As a rule their titers are very low and the reactions occur at low room temperature but not at 37 C Consequently these atypical agglutinins as a class play no role in the selection of compatible donors nor do they interfere with the scheme of the blood groups The differentiation of the subgroup A_1 and A is not always clear cut a number of bloods known as intermediates react with both anti A_1 and anti H Another important feature of these antibodies is the fact that all group O bloods react with anti H but not with anti A_1 The e relationships are shown in Table 17

Table 17 The Subgroups of Group A

	Tested With Blood			
Agglutinin	O	A_2	A_1	Intermediate
Anti A_1	o	o	+	±
Anti H	+	+	o	±

The differentiation of anti H from anti O in human sera may be made because the former are neutralized by saliva secretors of group O A or B while anti O is not neutralized Anti H is more frequently produced by nonsecretors and anti O by secretors The relationship of the secretor status to the Lewis system will be referred to below Anti H has also been *found in normal animal sera* (chicken beef rabbit) and may be produced experimentally by injection of goats or chickens with Shigellae bacilli vaccine or group O secretor saliva

A satisfactory reagent frequently employed for selection of A_1 and A red blood cells may be prepared by absorbing selected group B sera with A red cells These absorbed sera can be used at room temperature and are preferable to the naturally occurring anti A_1 which is rare low titered and inactive at room temperature

A still more potent anti A_1 may be prepared from selected group O or B donors or patients injected with group A specific substances or

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The rules of heredity follow

1 The dominant factors A or B cannot be present in the blood of the child unless it be derived from one or both of the parents

2 Combinations of group O parent with *group* AB child and *group* AB parent with group O child are excluded

The subgroups A_1 and A are also inherited but their use in cases of disputed paternity is not justified because their antibodies are not sufficiently potent to give sharp differentiation Forms intermediate between A_1 and A are known to exist and A_1 is not fully developed in the newborn infant It is not possible to differentiate *A 1* from *AO* because neither anti H nor anti O is specific for gene *O*

MN and Ss Factors

In 1927 28 Landsteiner and Levine described two serologically and genetically related blood factors with the aid of hetero immune rabbit sera prepared by the injection of human blood In the preparation of such antisera absorption with selected blood is necessary to render them free from species agglutinins The absorbed sera give 3 types of reactions called M MN and N A type of blood analogous to group O of the ABO system lacking both M and N does not exist The M and N factors are inherited as allelic genes of equal dominance so that the phenotype M (agglutinated only by an anti M serum) is homozygous for M (genotype *MM*) The MN system has been used extensively in cases of disputed paternity and by the use of these two antisera alone about 18 per cent of falsely accused men can be exonerated

More recently our knowledge of this system was increased by the discovery by Walsh and Montgomery of an antibody in human serum that was clearly related to the MN system This antibody later called anti S was thought to be inherited as a closely linked gene and its allele s was postulated Later this hypothesis proved to be correct when anti s was discovered by Levine et al The antisera defining the S s factors are found only in human sera and repeated attempts to produce them in rabbits have failed The iso agglutinin anti S is found more frequently in association with anti Rh but when present by itself it usually behaves as a naturally occurring agglutinin inactive at 37 C However antigenic stimulus (blood transfusion or pregnancy) increases its titer and thermal amplitude to make it clinically important

Using the 4 antisera—anti M anti N anti S and anti s—9 phenotypes corresponding to 10 genotypes can be distinguished Very weak forms of M and N have been reported but are very rare

The MN factors have been effectively employed in studies on the survival of transfused erythrocytes Such studies have confirmed the earlier observations of Ashby that the normal life span of the red cell is between 90 and 110 days Almost identical results have been obtained using radio chromium tagged red cells

Numerous examples of human anti M and anti N have now been found the majority occurring as cold agglutinins but antigenic stimuli from transfusions and/or pregnancy may result in the production of a clinically significant antibody Anti M is found far more frequently than anti N although Race and Sanger cite 10 examples of the latter Extracts of *Vicia graminea* and other Leguminosea have been shown to possess N specificity

Anti S which reacts with about 54 per cent of the caucasoid population has been reported frequently and has been incriminated as a cause of hemolytic disease of the newborn whereas anti-s is found much more rarely and always appears to be the result of immunization It reacts with 88 per cent of the population

The MNSs system is complicated by the occurrence of an antibody anti U which reacts on all bloods except the very rare blood lacking both S and s exclusively negroid Other antigens belonging to the MNSs system are Hunter and Henshaw especially among negroids also possibly 2 related low incidence factors Miltenberger (Mi) and Verweyst (V^w) may be included

ISO IMMUNIZATION AND THE Rh FACTOR

Historical

In the course of studies on the factor in rhesus blood related to but not identical with M Landsteiner and Wiener found still another relationship between rhesus and human blood i e the Rh factor The human Rh factor which is antigenic in man and causes hemolytic disease of the newborn was discovered in 1939 by Levine and Stetson with the aid of a human immune iso agglutinin

from group O mothers who delivered group A infants These sera when neutralized by the addition of group A specific substance will react specifically with A_1 red cells suspended in group *AB* serum (from a nonsecretor), or with saline suspended red cells followed by the antiglobulin reaction described below

Several varieties of group A (and group AB) bloods more weakly agglutinable than A have been described such as A_3 A_4 A A_m In general the A agglutinogen is more readily detected by the anti A of group O sera than by anti A of group B Therefore it is recommended that potent group O serum be included in all blood grouping tests in addition to the isolated anti A and anti B

Weak forms of B occur far more rarely They have been found in natives of Pakistan and in Negroes A genetically suppressed type of B was found in 3 members of one family—all nonsecretors The bloods behave like group O but their serum contains anti H Other group O bloods containing anti H have been described mainly in India (Bombay type)

Iso agglutinins Anti A and Anti B

The iso agglutinins present in the newborn are in the majority of cases derived from the mother by placental transmission These are replaced later by those antibodies characteristic of the blood of the infant The passage of iso agglutinins from mother to infant is greatest in the combination of group O mother and group *O child The titer varies during the* life of the individual and tends to diminish in old age and pathologic conditions such as leukemia and nephritis These antibodies are part of the globulin fraction of normal human serum Iso agglutinins have also been found in milk transudates and pus They are present in *purulent but not in normal cerebrospinal* fluid

All blood grouping sera must be standardized for avidity titer and specificity in accordance with the minimum requirements released in 1946 by the National Institutes of Health Blood grouping sera must be specific i e free *from all other agglutinins (cold or auto* agglutinins or other atypical antibodies) and they may not exhibit pseudo agglutination (rouleaux formation)

High titered anti A and anti B are best obtained by injecting volunteers of groups A and B with sterile purified groups B and A substance, *respectively* Potent antibodies may also be produced in rabbits or other suitable animals injected with group A or B blood but such sera which must be processed by absorption to render them *specific* lack *the* avidity found in human sera

Anti A and/or anti B of group O mothers have been shown to be responsible for many mild and occasionally severe cases of hemolytic disease of the newborn There are no certain serologic properties of these antibodies *to render them as diagnostic Incompatible* ABO matings also reduce the incidence of Rh hemolytic disease

Although not yet of practical importance extracts of certain plants (seeds) have specificities corresponding to anti A, such as Lima beans *Vicia cracca* and *Dolichus biflorus* The latter is almost specific for A_1 Anti B is found in extracts of the mushroom *Marasmius orcades* Anti H was found in *Ulex europeus*

Heredity of the ABO System

The blood groups are inherited as a series of 3 allelic genes, *O A* and *B* the latter two being dominant over *O* Accordingly there are 6 genotypes (*OO AO AA BO BB* and *AB*) and 4 phenotypes since the homozygous and the heterozygous forms of A and B cannot be differentiated from each other The rules of heredity for the 9 phenotypic matings and the blood groups of children possible in each of the matings are given in Table 18 Statistically speaking for each 100 instances of known false accusations of paternity tests for the ABO factors alone will suffice to exclude 16 to 17 per cent

Table 18 The Heredity of the ABO Groups

Matings Given as Phenotypes

Matings	Children Possible
O × O	O
O × A	O A
O × B	O B
O × AB	A B
A × A	O A
A × B	O A, B AB
A × AB	A B AB
B × B	O B
B × AB	A B AB
AB × AB	A B AB

TABLE 19 THE 3 RH HR SERA
Incidence of Reactions and Antibodies Produced

Terminology		Per Cent		
Fisher Race	Wiener	Positive	Negative	Antibodies
Anti D	Anti Rh	85	15	Very frequent
Anti C	Anti rh	73	27	Very rare
Anti c	Anti hr′	80	20	Occasional
Anti E	Anti rh	30	0	Occasional
Anti e	Anti hr	97	3	Very rare

liminary studies indicate that saline agglutinins do not pass from the mother to the infant and therefore are not responsible for hemolytic disease These antibodies have a larger molecular size than albumin agglutinins Saline (complete) agglutinins are found mainly in the gamma globulin fraction while albumin (incomplete) agglutinins are found in the gamma and other globulin fractions of human sera

As in the case of blood grouping sera the quality of anti Rh sera is now controlled by the National Institutes of Health Such sera must satisfy certain criteria as protein content avidity and specificity

A continuous supply of potent human anti Rh sera is obtained from Rh negative mothers of affected infants by antigenic stimulation to increase the titer and the avidity or by more prolonged stimulation of volunteer Rh negative donors Albumin agglutinins are produced far more frequently than saline agglutinins As a group these and related antibodies have been called warm iso immune agglutinins since their avidity and titer is greater at 37 C than at room temperature

THE SUBTYPES OF RH HR

The study of sera of the small group of Rh positive mothers of infants with hemolytic disease revealed a variety of antibodies specific for factors related to the Rh factor There are at least 2 other Rh factors in addition to Rh_0 and 2 corresponding Hr factors The term Hr a mirror image of Rh was chosen in order to indicate an allelic relationship analogous to that of M to N

The incidence of positive and negative reactions with the 3 Rh and the 2 Hr sera and their identification in terms of existing terminologies are given in Table 19 An antibody corresponding in specificity to anti d has not yet been confirmed

On testing all bloods with any 2 Rh sera 4 types are observed and each is split into 2 other classes on including the third Rh serum The incidence of the 8 types in the white population is given in Table 20 The 8 subtypes are further divided in terms of reactions with anti c (anti hr) and anti e (anti hr′) The factor d is given but cannot be identified because anti d is not available

In order to avoid undue complexity th several variants recently described are not included in Table 20 The variants of D are D mentioned above and D − a type of blood in which D is more reactive but fails to react with the antibodies for C c and E e systems The variants of C in order of their frequencies are C^w C C and c The E variants are E and E^w Antibodies exist only for

TABLE 20 THE 8 RH SUBTYPES

Terminology		Reactions With			Incidence	
Fisher Race	Wiener	Anti D	Anti C	Anti E	Per Cent	Classification
1 DC	Rh_1	+	+	o	54	Rh positive (85%)
2 DCE	Rh_1Rh	+	+	+	15	
3 DcE	Rh	+	o	+	14	
4 Dce	Rh	+	o	o	2	
5 dce	rh	o	o	o	13	Rh negative (15%)
6 dCe	rh	o	+	o	1 5	
7 dcE	rh	o	o	+	0 5	
8 dCE	rh rh	o	+	+	very rare	

produced by transplacental immunization The association of hemolytic transfusion reactions in Rh negative patients with the production of anti Rh was shown in 1940 by Wiener and Peters Accordingly rhesus blood contains a factor related to but not identical with the human Rh factor Experimental Rh sera produced in rabbits or other animals are now of interest only historically since all diagnostic sera now in use are derived from human sources

In almost all instances of intragroup transfusion reactions or of hemolytic disease of the newborn the recipient or the mother is Rh negative and has been immunized by the Rh factor in the donor s blood or fetal red blood cells, respectively Anti Rh_0 (anti D) gives 85 per cent positive reactions and 15 per cent negative reactions in the white population of the U S A The 2 types will be referred to as Rh positive and Rh negative respectively In contrast with anti A and anti B anti Rh is not found in the serum of Rh negative individuals unless they have been subjected to the antigenic stimulus of Rh positive blood by transfusion intramuscular injection or by *fetal blood via placental transfer*

It now becomes essential to carry out Rh tests in all candidates for transfusion so that all Rh negative patients receive exclusively Rh negative blood This is especially urgent in young girls or women prior to or during their childbearing age Rh tests are now a routine procedure in all prenatal cases for the selection of Rh negative mothers who deliver almost all infants suffering from intragroup hemolytic disease

Diagnostic Anti Rh_0 (Anti D) Sera

Differentiation of Rh positive and Rh negative individuals is readily made with standard potent and specific anti Rh antibodies These occur in 2 main forms (1) saline agglutinins which give direct reactions with saline suspended cells and (2) albumin agglutinins which although specifically combining with or coating saline suspended cells fail to produce the visible effect of agglutination However *albumin agglutinins* will give direct reactions with Rh positive cells suspended in human or bovine albumin, normal group compatible serum or still other media They also give direct reactions with enzyme *treated red* cells suspended in saline That albumin ag*glutinins specifically* combine with Rh positive cells suspended in saline is shown by the fact that such cells, like cord blood of the affected infant, will give direct reactions with suitably prepared rabbit antihuman sera (anti globulin reaction of Coombs) The antihuman serum unites specifically with the layer of *antibody which coats Rh positive red cells*

In the light of recent studies it is now possible to define the qualities of an anti human serum which will detect antibodies for the Rh Hr system and other antibodies outside this system The reagent must be capable of detecting the layer of specific globulin coating the red blood cell *whether this coating is* gamma globulin e g anti Rh or so called nongamma globulin " such as anti Le , or anti Jk For the latter variety, fresh complement containing serum appears to be essential if atypical antibodies are to be detected

If the specific union of antigen and antibody has occurred in vivo as in hemolytic disease or in acquired hemolytic anemia it can be detected by the direct Coombs reaction *In* the indirect Coombs reaction *the* specific union or coating occurs in vitro on the addition of albumin (nonsaline) agglutinins to saline suspended cells The indirect reaction is routinely employed *for the detec*tion of the so called D variety of Rh positives which reacts very weakly or not at all with some anti D saline sera so that frequently they are wrongly diagnosed as Rh negative The presence of the D^u antigen in such bloods is indicated by a strong indirect *reaction with antihuman serum*

Saline agglutinins are *heat labile frequently* appear early in the course of immunization and are termed complete ' antibodies Albumin agglutinins are heat stable appear later in the course of immunization and are referred to as incomplete ' antibodies Less frequently they have been designated as coating antibodies because in a saline medium they satu*rate the surface of the red cells which now* will fail to react with saline agglutinins Sera exhibiting this phenomenon are said to contain blocking antibodies Occasionally sera *containing albumin agglutinins* will show a prozone on titration The prozone cannot be demonstrated if the tubes are not allowed to incubate at 37° C but are read after im*mediate centrifugation at high* speed Pre

Aside from the determination of genotype the further analysis is required for the pre vention of iso immunization and intragroup transfusion reactions in Rh negative patients who may have produced antibodies in addi tion to anti D or prevention of transfusion accidents in Rh positive individuals immu nized either by transfusion and/or pregnancy In transplacental iso immunization of Rh positive mothers with hemolytic disease re sulting from the action of antibodies for C c or E e factors—mainly anti c and anti E— the genotype of the husband is readily deter mined

An indirect approach for the determination of the genotype is the so called double dose effect With selected sera mainly anti c and anti e bloods homozygous for the specific fac tor show considerably stronger reactions and higher titers than heterozygous bloods

OTHER BLOOD FACTORS

The Lewis System Le^a and Le^b

The Lewis system may be considered pri marily as one involving an antigen mainly in body fluids with secondary effects on the red blood cells

From 4 to 16 per cent of individuals lack Lewis antigens in either their body fluids or their red blood cells all other individuals possessing these antigens But uniquely the nature of the Lewis antigen found is dependent on the ABO (H) secretor status of the indi vidual

As mentioned above the ABO (H) secretor status is inherited as a mendelian dominant while nonsecretor is recessive and similarly Lewis secretor is dominant over Lewis non secretor According to Ceppellini the nature of the relationship of ABO (H) secretion and Lewis secretion is now believed to be due to gene interaction

In a simplified form these relationships are presented below

In tests of adult red cells with anti Le and anti Le^b, 3 types of reactions are observed as shown above The third type Le (a—b—), is highest in Negroes and can be divided into 2 subtypes based on ABO (H) secretion

Antibodies Anti Le^a and anti Le^b may be present as naturally occurring antibodies in the serum of an individual who does not secrete Lewis In the presence of sufficient antibody and complement specific hemolysis occurs and transfusion of incompatible blood has been responsible for hemolytic transfu sion reactions It is doubtful if anti Le has caused hemolytic disease of the newborn At times anti Le^b sera cannot be distinguished from anti H and frequently both antibodies coexist in the same serum

Further reference to Lewis antibodies will be made in the section on crossmatching

The Kell System (K k Kp Kp^b)

Probably the clinically most important fac tors aside from Rh Hr is the K k (Kell Cellano) system The antigen K occurs in 8 to 10 per cent of the random caucasoid popu lation and is unrelated genetically to any other known system Anti K is the cause of hemolytic disease of the newborn as well as transfusion reactions The Kell antigen is im munologically an extremely good antigen since persons receiving numerous blood trans fusions and hence probably Kell positive blood frequently produce anti K

The antigen k (Cellano) is present in 99 8 per cent of all bloods hence 0 2 per cent of random bloods is homozygous KK and capa ble of producing anti k Anti k is also capable of causing hemolytic transfusion reactions and hemolytic disease of the newborn

Recently the Kell system has become com plicated by the discovery of another pair of allelic genes closely linked to the K k locus Anti Penny (Kp) reacts with about 2 per cent of random bloods and anti Routenberg (Kp^b) with 99 9 per cent One example of anti Kp^a and four examples of anti Kp^b have been found

	ABO (H) Secretion 80%	ABO (H) Nonsecretion 20%
	Phenotype of red cells	
Secretion of Lewis 96 to 84%	Le (a−b+)	Le (a+b−)
Nonsecretion of Lewis 4 to 16%	Le (a−b−) (may produce anti Le + anti Le^b)	Le (a−b−) (may produce anti Le^b + anti H)

C^w C and E^w. The other variants are characterized by weaker reactions with standard anti C and/or anti E.

Two other blood properties for which antibodies have been described are f and V. With rare exceptions anti f reacts with all bloods containing factors c and e on the same chromosome. Anti V reacts exclusively with selected f positive bloods—mainly negroid.

The Heredity of the Rh Hr Factors

The two contrasting theories, namely, very close linkage at 3 loci or a series of multiple alleles at one locus, are illustrated below.

Multiple Alleles (Wiener)		Linkage (Fisher Race)	
R^0 R^1 R	r r' r	D	(d)
		C	c
		E	e

According to the linkage theory each gene determines the presence of a particular blood factor, while the multiple allele theory requires that a single gene determine the presence of 3 factors. From a practical standpoint, it does not make much difference which of the 2 theories is correct, since the 2 concepts are indistinguishable if the linkage is very close.

In tests with anti C and anti c bloods lacking the genetically related factor are homozygous, i.e., CC (20%) and cc (27%). The remaining are heterozygous Cc (53%). Similarly, the frequencies of the 3 genotypes EE, Ee and ee are 3, 27 and 70 per cent, respectively.

From a clinical viewpoint it is important to determine the genotype of the Rh positive husband of an Rh negative wife, genotype dd. If he is homozygous DD, all offspring must be Dd. If he is heterozygous Dd, the offspring in the mating $Dd \times dd$ will be 50 per cent Dd or Rh positive and 50 per cent dd Rh negative. Since anti d is not available to differentiate genotype DD from Dd, a presumptive diagnosis may be made in terms of reactions with antibodies for C c and E e factors. On a statistical basis an Rh positive blood of genotype CC is also of genotype DD, and an Rh positive blood of genotype EE is also most likely to be of genotype DD. The presumptive diagnosis of genotypes emerges from the consideration of the 8 genetic combinations of chromosomes bearing the Rh Hr genes and their incidence in the white population as given in Table 21.

Table 21. The Frequency of the Rh Chromosomes

Terminology		
Fisher Race	Wiener	Per Cent
DCe	R^1	40.76
dce	r	38.86
DcE	R^2	14.11
Dce	R^0	2.57
dcE	r'	1.19
dCe	r	0.98
DCE	R	0.24
dCE	r^y	very low

Thus, the genotype of Rh_1 individuals may be either DCe/dce (R^1r) or DCe/DCe (R^1R^1). As given, the latter is homozygous for both D and C, and serologically a blood of this genotype is characterized by its failure to react with anti c. This indicates that the blood is homozygous for C (CC) and by statistical correlation is presumed to be homozygous also for D (DD) because the combination DCe is far more frequent than dCe. Should the latter condition obtain, the genotype of the Rh_1 individual would be DCe/dCe, in which event the blood, homozygous for C as before, would be heterozygous for the clinically more important D factor. Incidentally, a blood of the rare genotype DCe/dCe may react weakly with saline agglutinins and will behave like the usual hereditary variety of D, which is more frequent in Negroes. With genes D and C in transposition, i.e., on different chromosomes, the reactivity of D is diminished—an example of gene interaction. This rule holds for all other chromosome combinations in which D and C are in transposition.

Similarly, most Rh_1Rh individuals are homozygous for D since the most common gene combinations transmitted by the two parents are DCe (R^1) and DcE (R). Although heterozygous for C, the blood is homozygous for the clinically more important D factor. Examples of a source of error in the genotype of bloods reacting like Rh_1Rh are the transmission from one or both of the parents of rare chromosomes such as Dce/dCE (R^0r^y), DCe/dcE (R^1r'), DCE/dce (Rr) or DCE/dcE (Rr'). At times the genotype can be ascertained by family studies.

TABLE 22 THE BLOOD GROUP SYSTEMS AND THEIR CLINICAL IMPORTANCE

	Occurrence of Antibody in Absence of Known Antigenic Stimulus*	Role in Hemolytic Transfusion Reaction	Role in Hemolytic Disease	
			Frequency	Severity
ABO	Always (hem)	Common	Frequent	Mainly mild
Rh	Very rare	Common	Common	All grades—mainly severe
MN {Anti M	Occasional	Very rare	1 or 2 cases	
MN {Anti N	Rare	Very rare	Not known	
S s U {Anti S	Rare	Rare	Rare	Variable—can be severe
S s U {Others	Never	Rare	Rare (anti s)	Variable—can b severe
P	Rare	Rare	Not known	
Tj	Always (hem)	Rare	Not known	
Lewis (Le)	Occasional (hem)	Occasional	Not known	
Lutheran	Rare	Occasional mild	Not known	
Kell	Never	Occasional	Occasional	All grades
Duffy	Not known	Rare	Very rare	All grades
Kidd	Not known	Rare	Very rare	All grades
Low incidence	Only two**	Rare	Rare	All grades
High incidence (Vel)	Unknown (hem)	Rare	Not established	

(hem) = property of serum to hemolyse
* e cept in agammaglobulinemia
** Verwey t (V^w) and Wright (Wr^a)

variety either enzyme treated or suspended in 20 to 30 per cent bovine albumin Incubation at 37 C will reveal the presence of warm immune iso antibodies All readings may be made at the end of 1 hour with the aid of a small hand lens and confirmed when indicated by microscopic readings

The identification of the antibody may be made by testing the selected sera with a panel of group compatible red cells previously chosen on the basis of their antigenic composition In this manner it should be possible to identify each of the several antibodies which may be produced by patients immunized by a series of transfusions Whenever possible the identification should be confirmed by specific absorption experiments

The specificity of the antibody responsible for hemolytic disease of the newborn may be anticipated on the basis of specific differences in the antigenic make up of the red blood cells of husband and wife Although the mating may be incompatible for a number of blood factors the patient responds as a rule by producing antibodies to only one factor in striking contrast with patients immunized by a series of random compatible transfusions

In hemolytic disease of the newborn antibodies specific for low incidence blood factors may be demonstrated by including the father s cells in the test panel provided that they are ABO compatible

COMPATIBILITY

No matter how efficient the compatibility test may be it is not possible to prevent immunization in patients receiving transfusions unless care is taken to give blood of identical antigenic composition It is obvious that ABO group compatible blood must be given Even in extreme emergencies it would be highly desirable to abolish the use of group O and to employ only group homologous blood It is now routine procedure that Rh negative recipients receive only Rh negative blood

In view of recent reports of the high incidence of anti c (anti hr) among persons receiving numerous transfusions it seems advisable to transfuse Rh positive persons lacking the c factor with blood *also lacking the*

The Duffy System (Fy^a, Fy^b, Fy)

Numerous examples of antibodies to the Duffy (Fy^a) factor have been reported though rarely without the presence of other antibodies, usually anti D

The antigenic stimulus in nearly every case has been an incompatible blood transfusion, while at least one example is thought to have been the cause of hemolytic disease

The Fy^a factor is present in 65 per cent of the white population and the related Fy^b in 82 per cent The first example of anti Fy^b was reported in a case of hemolytic disease Since that time several other examples of the antibody have been observed The Duffy system has recently become complicated by the discovery that 65 per cent of New York negroids fail to react with both anti Fy^a and anti Fy^b This type of blood is termed Fy The genetic and serologic explanation for this phenomenon is still to be supplied

The Kidd System (Jk^a, Jk^b)

The first example of anti Kidd to be described was thought to be the cause of hemolytic disease Numerous examples have since been reported and nearly all have occurred in serum which also contains anti D Occasional examples of hemolytic disease and transfusion reactions have been reported as the result of this antibody The factor Jk^a is present in 75 per cent of caucasoid bloods and the genetically related factor, Jk^b, has about the same incidence

Lutheran

The Lutheran factor Lu^a is present in 8 per cent of the caucasoid population Anti Lu is probably naturally occurring and may cause transfusion reactions, mainly of the mild variety Recently the genetically related factor Lu^b has been reported by Cutbush and Mollison and shortly after its discovery one more example of anti Lu^b was found

P and Tj^a

The factor P has been thought to be genetically independent of other blood group factors until the recent observation of Sanger that all Tj^a negative persons are also P negative This led to the discovery that suitably absorbed anti Tj sera also contained anti P Anti P is a natural antibody and traces of anti P are found in a large percentage of P negative individuals The factor is present in about 75 per cent of the caucasoid population and in 97 per cent of the negroids The factor Tj^a is absent in only a few of the hundreds of persons tested, hence even though the antibody is always found in the absence of the antigen, it is still of very rare occurrence Anti Tj^a is a hemolytic antibody and has been responsible for transfusion reactions

Recently, a potent anti P has been found in the sera of P negative patients suffering from echinococcus cyst disease P soluble substance has been demonstrated in hydatid cyst fluid These individuals produced more potent anti P active also at 37 C and one can more readily attribute severe hemolytic reactions to this variety of anti P in contrast with occasional mild reactions in other cases

Low Incidence Factors

There have been numerous reports of antibodies to antigens seemingly present only in one family, but their interrelationship to each other and to the better known blood factors is still to be established Three striking examples of these so called family factors were later found to be of a much higher incidence (Mi^a V^w and Wr^a) Mi and V^w now shown to be related but not identical seem to be related to the MNS system

Of special interest is the Diego factor (Di^a) antibodies for which have been found on three occasions each associated with hemolytic disease of the newborn Although Di has an exceedingly low incidence in caucasoids it has a considerable incidence in American Indians Chinese and Japanese with values varying from 3 to 45 per cent For a fuller discussion of other blood factors of both low and high incidence the reader is referred to Race and Sanger

Table 22 presents in summary the several blood group systems their interrelationships and source of their more important characteristics

Detection of Isoimmunization and Identification of Antibodies

In the screening of sera to find those that contain antibodies it is essential to employ a test blood which has as many of the antigenic components as possible In practice a mixture of 2 group O bloods of types Rh_1 and Rh is frequently employed These bloods may be further selected for the presence of the following factors K k S Jk^a and Fy^a All sera are then tested with the mixture suspended in saline followed by the indirect Coombs test, and with cells of the same

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No matter how efficient the compatibility test may be it is not possible to prevent immunization in patients receiving transfusions unless care is taken to give blood of identical antigenic composition. It is obvious that ABO group compatible blood must be given. Even in extreme emergencies it would be highly desirable to abolish the use of group O and to employ only group homologous blood. It is now routine procedure that Rh negative recipients receive only Rh negative blood.

In view of recent reports of the high incidence of anti-c (anti hr') among persons receiving many transfusions it seems advisable to transfuse Rh-positive persons lacking the c factor with blood also lacking the

c factor In the near future it may also be desirable to prevent iso immunization for the important Kell E and perhaps Duffy blood factors

Of primary importance however, is the precaution that no person will receive incompatible blood A sufficiently sensitive procedure properly carried out such as outlined below should detect all incompatibilities

TUBE 1
(High Protein)

2 drops patient s *fresh* serum
2 drops donor s cells (5% suspended in own serum)
2 drops 20 to 30% bovine albumin

TUBE 2
(Saline—Coombs)

2 drops patient s *fresh* serum
2 drops donor s cells (5% in own serum or saline)

1 Centrifuge both tubes immediately and read macroscopically for agglutination and/or hemolysis (Sera containing anti Le[a] will frequently hemolyze red cells in the presence of adequate complement) Incompatibilities to ABO Rh and perhaps Kell may be obvious at this point If no incompatibility is observed incubate both tubes 30 to 60 minutes at 37 C

2 Centrifuge tube 1 again and look for agglutination and/or hemolysis if negative examine microscopically

3 Perform the Coombs test on tube 2 using a properly standardized reagent and read with hand lens or concave mirror This last step will detect all antibodies which are best demonstrated by this procedure i e antibodies for Kell Fy Jk and Le In this respect it is important to use fresh (complement containing) serum

INDIVIDUALITY OF HUMAN BLOOD

With the recent discovery of numerous new blood factors the evidence for the individuality of human blood is almost complete It is now possible to detect more than 1 million different varieties of blood based on all the possible permutations and combinations of the blood factors mentioned above These refer to phenotypes or serologically differentiable types, while the number of different genotypes is much greater From a theoretical view point, this remarkable individuality of human blood is believed to have some bearing on the outcome of transplantation of skin and other organs in man Aside from identical twins and chimeras in nonidentical twins (resulting from placental anastomosis) skin transplants are not successful, even if the donor and the recipient belong to the same blood groups It is doubtful, however, if transplantation between random donor and recipient will be successful even if all their blood factors are identical Apparently, skin and other organs contain antigenic factors independent of red cell antigens The A and B blood factors have been identified in tissue cells but, with the possible exception of antigen D of the Rh system none of the numerous other red cell antigens has been demonstrated in the fixed system of cells

Nothing is known about the function of the numerous antigens in red blood cells except that they probably have profound evolutionary significance since no genes are neutral in selection Current studies suggest a possible association of ABO blood groups and secretion status in human disease Factors indistinguishable from A and B are present in the blood of higher apes only while factors related to A B, M N and Rh have a wider distribution in animal species Finally mention may be made of the differences in the incidence of the factors A B, M N S s and the Rh Hr system in various racial groups

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The Diphtheria Bacilli and the Diphtheroids

CORYNEBACTERIUM DIPHTHERIAE

Wilson and Miles (1955a) define this group of micro organisms as follows

Gram positive rod like forms arranged usually in a palisade Not acid fast Often with club shaped swellings at the poles, generally with irregularly staining segments or granules Nonmotile nonsporing Growing aerobically or under microaerophilic conditions but often capable of anaerobic cultivation Never forming gas in carbohydrate media in which they may or may not produce acidity They may or may not liquefy gelatin or serum Some species produce a powerful exotoxin Type species *Corynebacterium diphtheriae* (Figs 1 D and 2 D)

Although representatives of the corynebacteria are widely distributed in nature, many strains appear to be associated with the body surfaces and tissues of animals and man Certain species are of significance in veterinary pathology, but in human disease the group of closely related forms known collectively as the diphtheria bacilli (*C diphtheriae*) occupy a position of outstanding and unique importance

In many respects diphtheria represents the disease in which the bacteriologist may feel the pride of maximal achievement Most of the facts regarding it—etiology, mode of transmission mechanism of pathogenesis therapy and prevention—have been thoroughly elucidated Diphtheria once the leading cause of death among children has virtually disappeared as a disease from civilized communities As stated by Burnet (1953) Other diseases are more important causes of death and some have been just as carefully and extensively studied as diphtheria but no other common disease has been so successfully studied Difficulties in application of our knowledge as well as some remaining lacunae in the facts themselves nevertheless permit the disease to continue in certain regions and occasionally to assume formidable proportions

To a considerable degree the success realized in the understanding and the control of diphtheria results from the circumstance that it is a purely toxic disorder uncomplicated at least in general by invasion of tissues by the micro organism It may be considered as providing the pattern for the understanding of

* This chapter was originally written by the late J Howard Mueller It is doubtful whether the problems connected with cultivation of the diphtheria bacillus and diagnosis of diphtheria have ever been described so succinctly and so critically and these sections have been left largely as he wrote them It is well to recall that Mueller s pioneer studies on nutrition and toxin production of *C diphtheriae* represented the first truly successful and complete identification of the factors required for growth of a fastidious micro organism and lead to studies in the metabolism of pathogenic bacteria that had been virtually impossible previous to his work

a group of diseases such as tetanus and botulism which closely resemble it in mechanism of pathogenesis and of a further group including scarlet fever and gas gangrene in which specific toxins are responsible for an important element of the disease process For these reasons it seems appropriate to select diphtheria as the first of the infectious diseases to receive detailed consideration

HISTORY

Although not established as a distinct clinical entity until the 19th century diphtheria in epidemic form has without question existed since the earliest times Undoubtedly it was often confused with other pathologic conditions affecting the throat the mouth and adjacent tissues streptococcal or fungal infections Vincent s angina nutritional disturbances such as scurvy and others alone or superimposed upon diphtheritic infection must have presented extraordinarily perplexing problems in diagnosis to the early physician In spite of these difficulties certain symptoms uniquely characteristic of diphtheria especially the paralysis of the soft palate with resulting regurgitation of fluid through the nose in attempting to swallow indicate clearly that the disease existed in the 6th century and probably even then had been known for hundreds of years During the years 1735 1740 for example New England and the Middle Atlantic state were ravaged by a throat distemper which from its description was almost certainly diphtheria and may well have caused the death of more than 20 per cent of the entire population under 15 years of age in those regions where it occurred (Caulfield 1939) However it was not until 1826 that the French physician Bretonneau of Tours placed the specific clinical diagnosis of diphtheria on a reasonably firm basis and recognized its infectious nature The diphtheria bacillus was seen and described by Klebs in 1883 in smears from pseudomembranes from the throats of patients with the disease However it was Loeffler who a year later established the diphtheria bacillus as the etiologic agent and reproduced a similar disease in animals using the bacterium grown in artificial culture For this reason the diphtheria bacillus has often been referred to as the Klebs Loeffler or K L bacillus Loeffler made the important observation that both in fatal human cases and in laboratory animals the organisms could rarely, if ever be demonstrated except in the local lesion of the mucous membrane Although tissue damage occurred in many remote organisms such as adrenals liver and heart these invariably proved to be sterile This lead Loeffler to postulate the formation of a diffusible poison by the organisms His prediction was verified in 1888 when Roux and Yersin announced the discovery of diphtheria toxin Roux and Yersin demonstrated that injection of sterile culture filtrates of the diphtheria bacillus into laboratory animals caused a fatal disease with lesions similar to those produced by living organisms Two years later von Behring and Kitasato found that repeated sublethal doses of toxin partially detoxified with iodine trichloride caused animals to elaborate a substance antitoxin which was capable of specifically neutralizing the toxin By 1891 sera from immunized animals were being used in the treatment of diphtheria Thus the foundation was laid for a half century of specific serum therapy and prophylaxis not only of diphtheria but also of a variety of other infectious diseases

The methods for controlling diphtheria in populations by mass immunization followed logically from these early observations and from the development of quantitative methods for bio assay of toxin and antitoxin by Paul Ehrlich

Suggested by Theobald Smith in 1909 toxin neutralized by antitoxin was shown by Behring in 1913 to induce immunity safely in both animals and man and was applied on a large scale by Park (1922) for the protection of children A simple test for immunity by the intracutaneous injection of minute amounts of toxin was developed in 1913 by Schick making it possible to define more accurately the need for and the results of artificial immunization Finally Ramon in 1923 showed that formalin treated toxin *anatoxin* (now commonly called *toxoid*) possessed certain advantages as an immunizing agent over toxin antitoxin mixtures and this material in one form or another has been used for wholesale immunization especially of children in many communities Thus the knowledge and the tools appear to be at hand for eradicating diphtheria

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between the severity of the case and the types of organism found, which were consequently named *gravis* *mitis* and *intermedius* Although fairly general confirmation of the existence and the cultural properties of these types has been achieved it appears that there are either additional types not readily differentiated by the same criteria or occasional atypical or transitional forms It now appears certain that the 3 main types can be derived from one another by mutation There is by no means uniform agreement however as to the correlation of type with clinical severity This matter will receive further consideration in a later section of this chapter

While colonies of the various types differ in appearance depending on the tellurite formula employed it is a simple matter to learn the differentiation with any one of the media In general the *gravis* colonies are largest tend to be flat slate gray to black in color with a dull or matt surface The *mitis* colonies are usually blacker convex with glossy surface and somewhat smaller than *gravis* The *intermedius* colonies are minute pinpoint in size and vary from gray to black depending upon the medium Colonies of *C Hofmanni* resemble the mitis type in size and color but usually are sufficiently different to permit recognition by the experienced bacteriologist Beyond the colony characteristics on tellurite plates there are several further criteria which assist in classification Thus *gravis* strains ferment starch with the formation of acid whereas *mitis* and *intermedius* are without effect *Mitis* strain in general produce slight hemolysis on blood agar *intermedius* fails to do so *gravis* cultures give irregular results Certain differential features appear when the organisms are grown in broth *gravis* strains tending to form pellicles *mitis* to grow diffusely whereas *intermedius* develops as a finely granular turbidity settling to leave a clear supernatant Morphology likewise assists in the differentiation *gravis* cultures manifesting short forms with little metachromatic material *mitis* occurring as long granular organisms and *intermedius* showing barred club shaped forms

Diphtheria bacilli may be divided into groups according to their antigenic composition Doubtless because toxigenicity is by far the most important factor concerned in pathogenicity serologic classification of this group of organisms has not received intensive study Up to the present no clear cut relationship of immunologic properties to the 3 main mutant types has been found

For a more detailed discussion of the types and their significance see McLeod 1943

It is evident from the above that the nature of growth to be expected of a diphtheria bacillus in the usual bacteriologic bouillon the agar slope or plate blood agar plates etc will vary with the type of the organism and that no further attempt at detailed description would be profitable

Cultivation

C diphtheriae is primarily an aerobic organism and multiplies poorly if at all under strict anaerobic conditions It grows readily on most of the usual laboratory media containing peptones and tissue extractives On liquid medium most strains tend to grow as a waxy pellicle or veil on the surface The nutritional requirements have been thoroughly investigated by Mueller (1940) who has shown that most strains require a variable number of specific amino acids and a carbon or energy source which may be glucose or some other sugar an organic acid or alcohol In addition most strains are deficient in their ability to synthesize biotin nicotinic acid and pantothenic acid Under the optimal conditions of oxygen supply pH control etc used in toxin production growth equivalent to 10 Gm dry weight of certain strains of diphtheria bacilli can be attained per liter in liquid medium

For initiating growth from minute inocula for example on agar plates traces of oleic acid and some additional unidentified substance have been shown to be necessary (Cohen and Mueller 1941)

Loeffler s Medium The use by Loeffler of coagulated blood serum for the initial isolation of *C diphtheriae* from cases of diphtheria resulted in the belief that such a medium manifested selective growth promoting properties for the diphtheria bacillus enabling it to outgrow other bacteria occurring in the throat and giving it a normal and typical morphology While there is no question of the utility of Loeffler s medium for diagnostic purposes it seems in the light of present knowledge that its success is due largely to the fact

The most recent chapter in the history of diphtheria was opened by Freeman s discovery in 1951 that only those bacterial strains growing in close association with a particular temperate bacteriophage are capable of producing diphtheria toxin Strains which do not carry the virus are nontoxigenic

MORPHOLOGY

The characteristic feature of the corynebacteria from which they derive their name, is their varying diameter often broader at one end than the other, resulting in a ' club shape A further characteristic depends on an irregular distribution of protoplasm within the cell causing uneven absorption of certain dyes which result when suitably stained in a beaded or barred appearance of the organism This may be particularly noticeable in the case of certain strains of the diphtheria bacilli often accentuated by growth on special media and leading to the appearance of well defined polar bodies These deeply staining bands and beads have been variously named metachromatic granules, Babes Ernst bodies etc Recent evidence strongly suggests that these granules are mainly composed of highly polymerized polyphosphoric acid (Ebel 1952) Corynebacteria are gram positive nonspore bearing rods without flagella or capsules which vary in size from 2 to several micra in length and from 0 5 to 1 0 micron in diameter *Evidently because following cell division* the two resulting bacteria break apart sharply the distribution of organisms in a stained smear is relatively characteristic The individual bacilli form sharp angles with each other and have been variously compared with piles of matches Chinese letters or cuneiform characters, to which the frequent wedge shape of the cell lends further suggestion The occasional occurrence of true branching, which has been observed in the growth of *C diphtheriae* together with the irregularities of protoplasmic distribution previously mentioned, have been interpreted by some bacteriologists as tending to separate this group of organisms from the true bacteria and place it somewhat above them in the organizational scale

The keynote of morphology in this group is the wide variation seen among strains—variation which however remains within certain general limits While morphology can aid the bacteriologist in the laboratory diagnosis of diphtheria and in the detection of carriers final identification always must rest on further characterization of cultural and biologic properties of the organisms The existence of nontoxigenic diphtheria bacilli which are morphologically and culturally identical with toxigenic strains serves to underline the limitations of morphologic criteria in diagnosis

COLONIAL MORPHOLOGY AND TYPES OF CORYNEBACTERIUM DIPHTHERIAE

For many years it has been known that potassium tellurite in amounts which inhibit the growth of most bacteria has little effect on *C diphtheriae* A series of tellurite containing formulae have been proposed for diagnostic use in diphtheria, and by means of one of *these and incited by the occurrence* on the continent and later in England of an outbreak of unusually severe diphtheria McLeod Happold and their collaborators (Anderson et al 1931) found that it was possible to differentiate quite sharply between 3 types of *C diphtheriae* Correlation appeared to exist



FIG 22 Different types of *Corynbacterium diphtheriae* (*left*) intermedius (*center*) gravis (*right*) mitis Magnification × 760

membrane if present and from the area of inflammation It should be taken by the physician with good illumination of the area and sent to the laboratory with the least possible delay While the diphtheria bacilli are not especially delicate and may survive for many hours on a cotton swab they die out progressively and the interval between taking the culture and its examination should be no greater than necessary In the laboratory a Loeffler slant a blood plate and a tellurite plate are inoculated with the swab and a smear is prepared which may be stained with dilute fuchsin and examined for the organisms of Vincent s angina It is not recommended that any attempt be made to identify *C diphtheriae* in the direct smear Except in the hands of a bacteriologist of long experience in such diagnosis the chance of error in either direction is too great After 15 to 24 hours incubation the cultures may be examined—the blood plate for hemolytic streptococci and the tellurite plate for the gray or black colonies of diphtheria bacilli Frequently no growth whatever will be found on the tellurite plate Provided that the blood plate has shown the presence of viable bacteria on the swab this furnishes strong presumptive evidence that no diphtheria bacilli are present but the plate should be reincubated for 24 hours more and again examined before being reported finally as negative In examining the tellurite plate one must keep in mind the difference in appearance of the 3 types of *C diphtheriae* and especially the relatively inconspicuous nature of *intermedius* growth If colonies are present which suggest any of the types a smear stained with methylene blue will enable one to determine promptly whether or not they are corynebacteria of some sort If so their morphology together with the colony appearance will frequently make identification quite certain In case of doubt the somewhat different morphology of the organism on the Loeffler slant may be of assistance *C hofmannii* for example is sufficiently characteristic to make recognition relatively easy

Where considerable numbers of cases of infection due to a single type o *C diphtheriae* are occurring in a community the matter need be carried no further the bacteriologist will readily identify the strain and a final report may be made covering the presence of the diphtheria bacillus the hemolytic streptococcus and Vincent s angina

Organisms from the suspected individual case the possible carrier (except during an epidemic of a known type) perhaps the occasional refractory convalescent carrier, and from any other unusual source such as a skin lesion should be isolated for further identification and a test for toxigenicity carried out

It is usually desirable to restreak a second tellurite or blood agar plate from the growth on the initial tellurite plate and then to isolate the culture from a single colony to a Loeffler slant or other suitable medium The pure culture thus obtained is used to inoculate tubes of Hiss serum water to check the fermentative properties Ordinarily glucose sucrose maltose and starch will be found to be sufficient for this purpose A tube of nutrient broth and a blood plate (if necessary) may also be inoculated and will yield additional information regarding type Except for a special study no agglutinative classification need be attempted

The so called virulence test may be carried out in the following manner

The growth on Loeffler slants is emulsified with broth to give slightly turbid bacterial suspensions From 0 1 to 0 2 ml of each suspension is injected intradermally into one of the shaved side of a guinea pig Then the suspensions are placed in the icebox After 4 hours 500 units antitoxin is administered intraperitoneally and 30 minutes later the test suspensions are injected into corresponding sites on the opposite side of the guinea pig Inflammatory reactions may occur at both corresponding sites within 24 to 48 hours but only sites injected with a toxigenic strain *before* antitoxin was administered go on to develop characteristic necrotic lesions at 48 to 72 hours This method of testing for toxigenicity has the advantage that lesions caused by a single bacterial suspension before and after injection of antitoxin may be compared in the same animal In case it is necessary to test many isolates rabbits may be used and are equally satisfactory

Some nontoxigenic strains are capable of causing pyogenic local skin lesions in rabbits and guinea pigs which resemble staphylococcal infections Similar lesions may be observed

that it is a relatively poor medium on which the diphtheria bacillus grows reasonably well but *is not quite good enough for the average* streptococcus or pneumococcus Perhaps it is worth noting that rather widely divergent re sults will be obtained on Loeffler s medium both as regards excellence of growth and mor phology depending upon the type of serum used in its preparation Since formulae in the literature variously specify beef sheep or swine serum, a *certain lack of uniformity of* results with this medium may be anticipated Swine serum for example seems either to be deficient in a factor favoring the growth of certain strains of *C diphtheriae* or more prob ably to contain a substance which suppresses their growth (Snyder and Mueller 1940) The exclusive use of Loeffler s medium for culti vating *C diphtheriae* in most laboratories probably also explains the long delay in rec ognizing the 3 well defined types *gravis mitis* and *intermedius* which are readily differenti ated by cultural characteristics as described in the preceding section

Biochemical Reactions

The diphtheria bacillus typically ferments glucose and maltose producing acid but not gas Occasional toxigenic strains have been isolated which *ferment sucrose Such strains* can be differentiated from morphologically similar *C xerosis* only by their toxigenicity and by their ability to ferment maltose (Barksdale et al 1957) *Gravis* types fer ment starch and dextrin whereas *mitis* and *intermedius* are without effect on these car bohydrates Growth and fermentation occur more promptly if the carbohydrate is incorpo rated into Hiss serum water than when plain broth is used as substrate Failure to ferment should be recorded only when demonstrable growth has occurred In starch and dextrin the absence of glucose as an impurity must be assured by suitable controls Otherwise false positives will result The fermentation re actions of the 3 main types of *C diphtheriae* and of certain related Corynebacteria fre quently found in the nasopharynx or the throat of man are summarized in Table 23

Certain strains of *C diphtheriae* isolated from human cases resemble *gravis types in* that they ferment starch, but differ from most strains in their ability to liquefy gelatin *Formerly these proteolytic strains were clas* sified as *Corynebacterium ulcerans* Since many of the strains are toxigenic and produc true diphtheria toxin, it seems more reason able to classify them as variants of *C diph theriae* (Henriksen and Grilland, 1952)

Bacteriologic Diagnosis

Current views tend to place the responsi bility for diagnosing diphtheria upon the clini cian leaving for the laboratory the task of bacteriologic confirmation This practice has been adopted because of the risk to the patient of delay in the administration of antitoxin It is safer to err on the side of an occasional needless serum treatment than to lose time which can make the difference between re covery and death To a degree therefore the necessity for a very rapid diagnosis need no *longer be felt by the bacteriologist, although* the diagnostic method employed should be no more time consuming than is consistent with accuracy The method to be selected must also be readily applicable to recognition of the diphtheria bacillus in the convalescent case and the healthy carrier as well as from the occasional unusual source such as the conjunc tiva a skin lesion or wound diphtheria

The diagnosis depends upon the recognition of a diphtheria bacillus in material taken from the site of infection usually the throat It is important that the specimen be obtained care fully and that it represent material from the

Table 23

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its lethal effect In fully susceptible animals all types of tissue seem to be sensitive and the toxin can cause injury to muscle liver skin nerve etc Because of the effect of iron on toxin production and its relation to the bacterial cytochrome content it has been suggested that diphtheria toxin may act by interfering in some way with the functioning of the cytochrome system in susceptible tissues (Pappenheimer 1947) The demonstration by Pinchot and Bloom (1950) of a depletion of phosphocreatine and decreased muscle organic phosphate in intoxicated guinea pigs is in keeping with this theory More convincing evidence has come from studies on the effect of the toxin on the cecropia silkworm at different stages in its metamorphosis (Pappenheimer and Williams 1952) Adult development of this insect a process which depend on rapid cytochrome synthesis is brought to a prompt standstill by injection of small amounts of diphtheria toxin The only other substances having a similar effect on the insect are known inhibitors of cytochromes The insect is highly resistant to the toxin during the pupal stage when the complete cytochrome system is not functioning

Toxigenicity and Lysogenicity

It was shown by Freeman (1951) that when nontoxigenic strains of *C diphtheriae* are treated with a particular bacteriophage a certain proportion of the resistant survivors upon plating give rise to colonies of toxigenic bacteria These survivors are lysogenic they carry a latent virus the prophage β In cultures of the converted toxigenic organisms a small proportion (about 1 in 50 000 cells) *spontaneously lyses to release mature* β phage which can then infect and convert new sensitive cells to toxigenicity and lysogenicity If the culture is induced by treatment with ultraviolet light almost all of the bacteria lyse with release of phage The studies of Groman (1953) and of Barksdale (1954) suggest that all toxigenic diphtheria bacilli are lysogenic for β phage or one of its variants Other diphtherial phages exist which are capable of lysogenizing but they do not convert sensitive strains to toxigenicity

Groman (1955) has shown that cured diphtheria bacilli from which β prophage has been eliminated are no longer able to produce toxin The cured strains are again sensitive to β phage and lysogenization of them again results in toxigenicity This type of alteration *of a bacterial genome brought about by the* mere presence of a particular prophage is known as *lysogenic conversion* This phenomenon differs from *transduction* where the bacterial genome becomes altered by the introduction of new *bacterial* genetic material through the agency of phage The acquired property in the case of transduction is controlled by bacterial genes whereas in lysogenic conversion the new attribute is apparently under the control of prophage

The close relationship between phage β and toxigenicity means that toxin formation can no longer be considered as a necessary criterion for the identification of *C diphtheria*

Pathogenicity for Animals

Natural infection of the lower animals with *C diphtheriae* appears not to occur However a variety of experimental animals are susceptible to the effects of its toxin and it has been possible to establish with the organism experimental infections which simulate the human disease Thus Loeffler in his early experiments was able by intratracheal inoculation so to infect rabbits and pigeons as to obtain *typical diphtheritic pseudomembranes at the* site of injury Indeed similar results had been observed earlier following inoculation of these animals with infectious material taken directly from human lesions (Wilson and Miles 1955b)

One of the characteristic features of many bacterial toxins is a marked variability in degree of action on different species of animals There is almost always a well marked selectivity evidenced by a relative susceptibility of certain species and the comparative resistance of others Within a single species however and excluding specific antitoxic immunity resulting from experimental procedure susceptibility is generally much more uniform

Thus susceptible human beings monkeys rabbits guinea pigs and horses all appear to be equally sensitive to diphtheria toxin when toxicity is expressed in lethal doses per unit weight On the other hand rats and mice are at least 1 000 times less susceptible The explanation for this species difference is not known and certainly is not due to antitoxin

with certain toxigenic strains even in control animals previously given antitoxin However, such pyogenic lesions are easily differentiated from the larger, hemorrhagic and necrotic lesions produced by toxigenic strains in the test animals

An in vitro method for testing toxigenicity has been described by Elek (1948) which gives results in good agreement with the guinea pig virulence test

Peptone maltose lactate agar containing 20 per cent horse serum is poured into a petri dish, and a strip of filter paper impregnated with antitoxin is placed across the plate After solidifying, 3 or 4 cultures to be tested are inoculated, each in a heavy streak at right angles across the paper strip After 48 hours of incubation antitoxin diffusing from the paper has encountered and formed a line of precipitate with toxin diffusing from positive cultures yielding 4 lines of visible precipitate radiating from the intersection of growth and paper For details the original article should be consulted It is of course essential that the antitoxin used contain no significant amounts of other diphtherial antibodies

Production and Properties of Diphtheria Toxin

Since the clinical injury caused by *C diphtheriae* is almost entirely accounted for by the toxin which it forms and since control of the disease depends on obtaining high yields of toxin for conversion to toxoid the production and the properties of this substance have practical as well as theoretical interest In order to obtain high yields of toxin in artificial media it is essential to select a suitable strain Different strains of *C diphtheriae* vary widely in their toxin producing capacity both *in vivo* and *in vitro* Park and Williams isolated an atypical strain in 1898 from a mild case of diphtheria which they found produced a more potent toxin than other strains which they studied The PW8 strain has been adopted by most laboratories for routine production of toxin Under proper conditions this strain is capable of releasing an amount of toxin equivalent to nearly 5 per cent of the bacterial mass As a further requirement for producing toxin in high yield it is essential that conditions be such that maximal growth is obtained The optimal conditions for growth and toxin production of the PW8 strain have been worked out by Mueller and Miller (1941) (see also Drew and Mueller, 1951) Finally, the most important single factor controlling toxin production seems to be the iron concentration in the medium (Pappenheimer and Johnson 1936) A certain minimum iron concentration is required for growth to occur When iron is added to an otherwise sufficient medium, growth and toxin production increase proportionally until the medium contains about 100 μg iron per liter At this concentration, toxin production is maximal but further addition of iron results in decreased toxin formation and almost no toxin is formed when 500 to 600 μg iron per liter is present Iron concentrations in this range and frequently higher occur in tissues, in the usual constituents of bacteriologic culture medium from dirty glassware and utensils and from a variety of unsuspected sources It is clear that many of the older statements on factors concerned in toxin production have little meaning because of failure to recognize and control the iron content of the medium

Kinetic studies have revealed that toxin is synthesized and released by cells only when their growth becomes limited by iron and as their cellular iron content begins to decrease One of the peculiarities of the PW8 strain which may well explain its high toxigenicity is its ability to increase 5 to 6 fold in mass after the exogenous iron supply has become completely exhausted (Yoneda, 1957 Pappenheimer 1955) At the same time the intracellular cytochrome content falls to ⅕ or ⅙ and an equivalent amount of free coproporphyrin III is liberated into the culture supernate together with the toxin These observations have lead to the suggestion that diphtheria toxin may be related in some way to cytochrome b the major respiratory pigment of the PW8 strain (Pappenheimer 1955)

Diphtheria toxin has been isolated on numerous occasions as a highly purified heat labile protein of molecular weight 72 000 which is homogeneous by the usual physiochemical criteria Recently Pope and Stevens (1955) have obtained the toxic protein in crystalline form The purest preparations are lethal for susceptible animals in doses of less than 0 2 μg per kilo of animal

It is not known how diphtheria toxin causes

acutely inflamed appearance swelling and pain of a streptococcus infection and has a relatively low fever of from 100 to 102 However he usually manifests a degree of prostration out of all proportion to the fever and the visible difficulty in the throat The membrane may spread with considerable rapidity over the tonsils the uvula and the posterior pharynx The dull white color gives place to a dirty gray and later to brown or in some instances black as a result of hemorrhage Separation of the membrane by mechanical means during the early stages uncovers bleeding points and is rapidly followed by the formation of fresh exudate

The cervical glands early become swollen and tender and in the severe or bullneck variety there is a massive edema of the tissue of the neck and the chest If the membrane developed initially in the larynx or if it extends to that site and continues further into the trachea death may result from mechanical stoppage of the air passage unless promptly relieved by intubation or tracheotomy Excluding such mechanical termination of the infection and in the absence of antitoxin treatment the patient will run the natural course of the disease and die during the acute stage as a result of general toxic effect succumb after a somewhat longer time as a result of cardiac damage by the toxin or recover after perhaps showing evidence of neurotoxic injury such as paralysis of the soft palate the ciliary muscles of the eye or the extremities The membrane separates after a few days and is eliminated leaving as a rule very little ulceration of the underlying tissues

The infection may occur initially in the ear or in the anterior nares and in the latter site particularly is likely to be relatively mild though prolonged We have seen a fatal case in a child in which the membrane was located in the umbilicus Rarely the conjunctiva may become the site of extremely severe and destructive diphtheritic lesion

Skin or wound diphtheria is rarely seen under ordinary conditions in temperate climates In the tropics however it is apparently not uncommon and indeed presented the armed forces with a rather serious problem in Africa and in portions of the Pacific theater during World War II The infection appears usually the site of some relatively minor injury a bruise a scratch or a blister and develops as an ulcer showing little tendency to heal with sharply demarcated edge and a dirty grayish slough or membrane covering the base Such infections may last for weeks or months and cover areas of several centimeters diameter Their nature may be unsuspected because of the rarity of the condition under normal circumstances and in temperate climates Conflicting reports are recorded regarding the type of flora and the virulence status of the diphtheria bacilli obtained in culture but the consensus appears to be that virulent organisms of the *mitis* type are generally involved Absorption of toxin is apparently minimal since few fatalities although a not inconsiderable number of late paralyses have been observed Response to antitoxin seems not to be uniformly favorable and it is possible that the etiology of the condition is complicated by some other factor

The pathology of the fatal human case of diphtheria except for the more obvious local picture is not particularly striking Evidence is seen grossly of toxic degenerative change in heart liver kidneys and adrenals which consist microscopically of parenchymatous degeneration fatty infiltration and necrosis It is not possible on the basis of these observations to reach any conclusion as to specific sites of action on the part of the toxin The cardiac damage one of the very common causes of fatal termination was believed for a time to be due to vasomotor disturbances in circulation but it now appears that actual injury to cardiac muscle sometimes involving the conducting mechanism may be involved equally well The lesions in the adrenal are much less conspicuous than in the guinea pig but MacCallum states that these organs are likely to show more prominent degenerative changes than the other viscera

It is well recognized that some strains of *C diphtheria* are capable of causing a more severe disease in man than other strains The studies of McLeod (1943) and his co workers have indicated that the more severe and fatal outbreaks of the disease are due frequently to the *gravis* type *intermedius* occupies the position of next importance *mitis* is found most frequently in endemic areas The first two types tend to produce the more toxic

It is possible that some kind of permeability effect may account for the resistance of rat and mouse cells since the same type of tissue damage occurs as in guinea pigs, provided that a sufficiently high dose of toxin is given A similar explanation may account for the fact that cold blooded vertebrates such as amphibia and reptiles are resistant to toxin at lower temperatures but become susceptible when their body temperature is raised above 25° or 30° C (Grasset and Zonderdyk 1931) With the exception of certain insects as cited above the protozoa invertebrates and plants have proved to be wholly resistant to the toxin

Injection of diphtheria toxin subcutaneously into an animal such as the guinea pig is followed in a few hours by local swelling and apparent tenderness Death ensues in from 24 hours to several days depending upon the amount of toxin given With suitably gauged sublethal doses late paralyses may occur resembling those seen in man during convalescence from diphtheria Examination at autopsy following death from a fatal dose reveals intense edema of the subcutaneous tissue at the site of injection often hemorrhagic in character Beyond this the most constant and striking pathologic picture is the marked congestion of the adrenal cortices frequently accompanied by hemorrhage Occasionally hemorrhage in the pericardium or the diaphragm occurs and the heart muscle the liver and the kidneys may show fatty degenerative changes By definition a minimum lethal dose (M L D) is that amount of diphtheria toxin that will kill a 250 Gm guinea pig on the 4th or 5th day following subcutaneous injection

If cultures of virulent diphtheria bacilli are injected rather than toxin itself the chain of events and the pathologic findings follow closely on those observed after the administration of the toxin The bacilli themselves as Loeffler recognized remain localized and in general are found at autopsy only in the vicinity of the site of inoculation Positive cultures occasionally obtained from the viscera appear to be due to terminal or postmortem invasion rather than attributable to any active invasive ability on the part of the organism

In the diphtheria bacillus therefore we have an organism possessing in some degree the ability to survive and to establish itself in the healthy tissues of a susceptible animal to an extent sufficient for the elaboration of an amount of toxin which is capable of causing death of the animal through specific and distant action on certain kinds of tissue This property, common to all 'virulent strains of *C diphtheriae* is lacking in 'nonvirulent strains which may otherwise be indistinguishable from the former group

In considering the significance of so called "virulence tests at least three factors should be borne in mind (1) the amount of preformed toxin in the inoculum, (2) the degree to which local proliferation of the injected organism takes place and (3) the rate at which it elaborates toxin during growth At the present time there seems to be no way of quantitating these factors

Diphtheria in Man

The diphtheria bacillus occurs in nature so far as is known only in lesions of the specific disease in man and in the throats and the noses of the normal human carrier From one of these two sources a virulent diphtheria bacillus reaches more or less directly—by droplet contact or fomites—the throat of a susceptible individual Growth presuambly is initiated in a superficial layer of mucus and desquamated epithelial cells and small amounts of toxin are elaborated This toxin absorbed into adjoining living cells destroys them in a few hours through its local necrotizing action The nidus of necrotic tissue supplies favorable conditions for further growth of the organisms more toxin is formed, and the process extends both laterally and more deeply into the tissue

Meanwhile there is an inflammatory reaction on the part of the body, capillaries engorge leukocytes enter red cells become extravasated and a layer of exudate begins to form which is composed of all these various elements This is at first grayish and inconspicuous but as the process continues it soon becomes thicker and tough forming a dull white layer or pseudomembrane covering the area The initial lesion may cover a tonsil or a portion of the posterior pharynx In some cases it is limited to the posterior nares or the trachea and thus may elude observation At this stage of the disease the patient has typically a moderately sore throat lacking the

acutely inflamed appearance swelling and pain of a streptococcus infection and has a relatively low fever of from 100 to 102 However he usually manifests a degree of prostration out of all proportion to the fever and the visible difficulty in the throat The membrane may spread with considerable rapidity over the tonsils the uvula and the posterior pharynx The dull white color gives place to a dirty gray and later to brown or in some instances black as a result of hemorrhage Separation of the membrane by mechanical means during the early stages uncovers bleeding points and is rapidly followed by the formation of fresh exudate

The cervical glands early become swollen and tender and in the severe or bullneck variety there is a massive edema of the tissue of the neck and the chest If the membrane developed initially in the larynx or if it extends to that site and continues further into the trachea death may result from mechanical stoppage of the air passage unless promptly relieved by intubation or tracheotomy Excluding such mechanical termination of the infection and in the absence of antitoxin treatment the patient will run the natural course of the disease and die during the acute stage as a result of general toxic effect succumb after a somewhat longer time as a result of cardiac damage by the toxin or recover after perhap showing evidence of neurotoxic injury such as paralysis of the soft palate the ciliary muscles of the eye or the extremities The membrane separates after a few days and is eliminated leaving as a rule very little ulceration of the underlying tissues

The infection may occur initially in the ear or in the anterior nares and in the latter site particularly is likely to be relatively mild though prolonged We have seen a fatal case in a child in which the membrane was located in the umbilicus Rarely the conjunctiva may become the site of extremely severe and destructive diphtheritic lesion

Skin or wound diphtheria is rarely seen under ordinary conditions in temperate climates In the tropics however it is apparently not uncommon and indeed presented the armed forces with a rather serious problem in Africa and in portions of the Pacific theater during World War II The infection appears usually the site of some relatively minor injury a bruise a scratch or a blister and develops as an ulcer showing little tendency to heal with sharply demarcated edges and a dirty grayish slough or membrane covering the base Such infections may last for weeks or months and cover areas of several centimeters diameter Their nature may be unsuspected because of the rarity of the condition under normal circumstances and in temperate climates Conflicting reports are recorded regarding the type of flora and the virulence status of the diphtheria bacilli obtained in culture but the consensus appears to be that virulent organisms of the *mitis* type are generally involved Absorption of toxin is apparently minimal since few fatalities although a not inconsiderable number of late paralyses have been observed Response to antitoxin seems not to be uniformly favorable and it is possible that the etiology of the condition is complicated by some other factor

The pathology of the fatal human case of diphtheria except for the more obvious local picture is not particularly striking Evidence is seen grossly of toxic degenerative change in heart liver kidneys and adrenals which consist microscopically of parenchymatous degeneration fatty infiltration and necrosis It is not possible on the basis of these observations to reach any conclusion as to specific sites of action on the part of the toxin The cardiac damage one of the very common causes of fatal termination was believed for a time to be due to vasomotor disturbances in circulation but it now appears that actual injury to cardiac muscle sometimes involving the conducting mechanism may be involved equally well The lesions in the adrenals are much less conspicuous than in the guinea pig but MacCallum states that these organs are likely to show more prominent degenerative changes than the other viscera

It is well recognized that some strains of *C diphtheria* are capable of causing a more severe disease in man than other strains The studies of McLeod (1943) and his co workers have indicated that the more severe and fatal outbreaks of the disease are due frequently to the *gravis* type *intermedius* occupies the position of next importance *mitis* is found most frequently in endemic areas The first two types tend to produce the more toxic

forms of the disease with a correspondingly greater mortality than *mitis* infections Fatality with the *mitis* strain is believed to be due more often to tracheal or laryngeal obstruction than to toxic injury

When a well agitated culture even of a highly toxigenic strain is grown in an artificial medium with an iron content equivalent to that in a diphtheritic membrane, no toxin is produced However, it should be emphasized that such conditions are highly artificial Diphtheria bacilli growing in vivo on mucous membranes are undoubtedly clumped together and are probably not in equilibrium with their nutritional environment There will be a competition for nutrients including iron diffusing out from the tissues It is likely that the toxin actually released in vivo arises from the relatively small proportion of bacteria that fail to receive their full share of iron The severity of the clinical disease that a given strain can cause in a fully susceptible individual will depend on the rapidity and the amount of toxin it can form in vivo Doubtless this will depend in turn on the speed with which the organisms can establish themselves to form the local lesion As yet unidentified factors may determine the extent and the rapidity with which local invasion is accomplished

Treatment

Since diphtheria is essentially a toxemia with very little invasion of the tissues by the organisms themselves it appears that prompt recovery should follow administration of the specifically neutralizing antitoxin Unfortunately the problem is not quite so simple Diphtheria toxin in the test tube it is true is neutralized promptly completely and multiple for multiple by antitoxin Although it can be shown that the toxin is not actually destroyed the union is a relatively firm one and dissociation apparently does not occur to a significant degree if the complex is injected into the animal body However free toxin when introduced into the circulation evidently attaches itself promptly and firmly to some of the body cells It is not known whether the ability so to combine with toxin is an attribute of a certain cell type or of some specific component common to various cells In any event when this union has taken place, the toxin seems no longer to be subject to neutralization by antitoxin and the train of events which the toxin is capable of initiating continues even in the presence of a considerable excess of antitoxin in the circulation Thus if a series of animals be injected with similar quantities of diphtheria toxin, the animals being divided into groups and varying amounts of antitoxin administered immediately to one group, after 5 minutes to a second group and so on it is found that the amount of antitoxin necessary to save life increases very rapidly with the time interval The following tabulation, cited by Zinsser et al (1939) shows the amounts of antitoxin necessary to prevent death in rabbits at various intervals after the administration of 10 fatal doses of toxin It is evident that after a delay of 90 minutes no amount of antitoxin will save the animals

Given after 10 minutes	5 units antitoxin
Given after 20 minutes	200 units antitoxin
Given after 30 minutes	2 000 units antitoxin
Given after 45 minutes	4 000 units antitoxin
Given after 60 minutes	5 000 units antitoxin
Given after 90 minutes	No amount

The exact explanation of the failure of toxin to be neutralized by antitoxin after attachment to the tissues is not yet known There are, of course, several possibilities Perhaps the antitoxin is unable to enter the body cells into which the toxin may pass The toxin may combine with the cell by the same bonds with which it unites with antitoxin these being satisfied there is nothing left to bring about the union

Finally perhaps the toxin brings about some change in the cell almost as soon as it becomes attached and the injury being accomplished it does not matter that the toxin molecule is then neutralized Whatever the ultimate explanation, and as pointed out in an earlier section, its understanding may well be of great significance the fact itself supplies the guiding principle which must be applied in antitoxin therapy *Treatment must be prompt and adequate*

The physician seeing a patient whose throat suggests the reasonable probability of diphtheritic infection should send a culture to the laboratory but should administer antitoxin at once The laboratory report will later confirm or refute the diagnosis but far less damage

will be done by the administration of an occasional unnecessary dose of antitoxin than by delay in its use when it is required The following tabulation of fatality according to the day of the disease on which antitoxin was administered is quoted by Russell (1943)

TABLE 24

ANTITOXIN GIVEN ON	CASES	CASE FATALITY
1st day of disease	225	0
2nd day of disease	1 441	4 2
3rd day of disease	1 600	11 1
4th day of disease	1 276	17 3
5th day of disease and upward	1 645	18 7

It is important that no chemotherapy b administered before taking the culture Failure to observe this precaution may lead to false negative results or prolonged delay in growth of the organisms

No definite rule can be laid down for the amount of antitoxin required for adequate therapy The actual amount of toxin which has gained entrance to the tissues on the first or second day of the disease is probably small and could be expressed in terms of a relatively few guinea pig minimal lethal doses Were this not so the mortality in untreated diphtheria would be 100 per cent Since 1 unit of antitoxin will neutralize (in the test tube) about 30 M L D it might appear that from 100 to 1 000 units would be more than adequate to obtain the desired effect However since it is known from experiments such as those mentioned above that within limit increasing the antitoxin will save life even after contact of the toxin with the tissue there is a reasonable basis for the current practice of treating diphtheria with relatively large doses of serum The objective should be to counteract as much as possible of the injury already done and to prevent further absorption of toxin Antitoxin is eliminated slowly so that a large single dose raising the blood level as high as possible will assure the maximum immediate therapeutic effect and provide a level in th blood for many days which will be adequate to cope with further toxin as it is ab orbed slowly from the local process in the throat

Various figures for the recommended unitage of antitoxin are to be found in the literature Perhaps one may state the general average as suggesting 100 units per pound body weight in mild cases up to 5 times that amount in the severe forms as adequate therapy It should be administered in a single dose intravenously except in the very mild case where intramuscular injection is satisfactory Of course skin tests for sensitivity to the protein of the antitoxin preparation must be carried out and where necessary desensitization must be undertaken

THE DIPHTHERIA CARRIER

Patients convalescing from diphtheria continue to harbor the organisms in the nose or the throat for a variable length of time following recovery The accompanying table modified from Russell (1943) shows that diphtheria bacilli can be isolated from the nose or from the nose and the throat in nearly 50 per cent of the cases during the 4th week of convalescence and that the bacilli are still present in a significant proportion during the 10th week The table suggests that an appreciable number of carriers will be missed if only throat cultures are tested for diphtheria bacilli

TABLE 25 PERCENTAGE OF CLINICAL CASES CARRYING DIPHTHERIA BACILLI DURING WEEKS OF CONVALESCENCE

WEEK OF CONVALESCENCE	NOSE OR NOSE AND THROAT (1 240 CASES)	THROAT (1 726 CASES)
0	100	100
2	76 0	50 1
3	63 1	35 3
4	48 3	20 6
6	24 6	7 6
8	12 6	2 6
10	4 8	0 7

The treatment and the disposition of these chronic carriers becomes then a most troublesome problem both scientifically and administratively and one for which no satisfactory solution has been found In many communities the health regulations require a continuation of quarantine of the individual until negative throat cultures are obtained on 3 consecutive days as a result considerable hardship is

suffered by the patient and the hospital also may find itself in a difficult situation Experience has shown that surgical correction of nose and throat abnormalities tonsillectomy etc coupled with mild gargles and mouth washes will result in some instances in clearing up the condition The use of bactericidal sprays and washes has been without effect At present evidence is accumulating that combined treatment of the case with antitoxin and penicillin in which the administration of the latter is continued for a time into convalescence leads to a much more rapid disappearance of positive cultures than was formerly true Opposed to this is the fact that some observers find diphtheria bacilli reappearing in the throat within a few days after interrupting the penicillin therapy It is therefore still premature to assume that a permanent cure for the convalescent carrier problem has been achieved

It is clear that circulating antitoxin does not prevent the establishment of the carrier state While avirulent *nontoxigenic* bacilli have been isolated from the throats of susceptible (Schick positive) persons the serum of healthy carriers of *toxigenic* strains has been shown invariably to contain an appreciable titer of antitoxin During the 1920 s the carrier rate for diphtheria bacilli in a large city such as London was estimated to be from 2 to 5 per cent and in institutions the rate was often found to be even higher (Russell 1943) It was predicted that with the advent of widespread immunization the carrier rate would rise still further Actually the reverse occurred and the almost universal immunization of urban children with toxoid has been accompanied by almost complete disappearance of the diphtheria bacillus and of the carrier state

Immunity and Epidemiology

In its basic features immunity to diphtheria is probably the simplest concept in the whole field of the study of specific resistance to disease Unfortunately in certain details of theory as well as of application difficulties are encountered

In the absence of previous contact with the diphtheria bacillus or its toxin (excluding the passive transfer of antibodies from immune mother to fetus through the placenta) the human infant possesses no neutralizin ability for diphtheria toxin and is therefore susceptible to infection when brought in contact with the diphtheria bacillus and presumably would retain this state of susceptibility indefinitely into later life One might postulate a type of nonspecific resistance which could be effective in certain individuals under these conditions such as a particular bacterial throat flora which provided unfavorable conditions for the growth of diphtheria bacilli should they find their way to it This sort of mechanism, though never demonstrated may exist occasionally but has no relation to the type of immunity under discussion Excluding some nonspecific protective mechanism, infection of such an individual with a virulent diphtheria bacillus would quite surely result in an attack of clinical disease If the organism were of high virulence and specific treatment were not available the individual would die within a few days since even under the most favorable conditions the body probably cannot initiate effective antibody formation in less than a week However if the virulence of the infecting strain were only moderate a nonfatal illness might result and in the case of a practically nonvirulent organism perhaps no discomfort whatever would be experienced In either of the two latter events the tissues would have experienced contact with diphtheria toxin although in extremely small quantity A single exposure to minute amounts of toxin extending over a period of a few days might serve as a sufficient stimulation to the tissues to form antitoxin in quantity which could be detected experimentally and would render the individual immune at least for the time being to further infection Much more probably it would result in nothing more than a conditioning of the tissues in such a way that later contact with diphtheria toxin would cause a much more effective antitoxin response to take place

Two points should now be evident (1) recovery from an attack of diphtheria need not result in immunity either permanent or transient and (2) repeated encounters with organisms of low virulence without evidence of disease may result in a fairly solid state of specific resistance to infection with a virulent strain Before the advent of antitoxin

and in communities where diphtheira was endemic with periodic occurrence of epidemics this probably represented in a general way the picture of the immunity in the population Diphtheria was primarily a disease of children and there was a fairly effective level of immunity in adults Therapeutic antitoxin p rhaps did little to alter this picture except to prevent a considerable number of deaths which otherwise would have occurred

It must be kept in mind that the above course of events takes place only because diphtheria bacilli of all grades of virulence are constantly present in a significant proportion of the population and diphtheria infection is relatively common If diphtheria were to disappear from a community the dissemination of virulent organisms from case to contacts would cease Lacking the stimulus for further antitoxin formation such a community would over the years lost its status of adult immunity and become uniformly susceptible at all age levels

As we have already seen the introduction of artificial immunization of large populations against diphtheria apparently has been an important factor in bringing about a substantial reduction in the prevalence of diphtheria bacilli In some communities the organisms seem to have disappeared almost entirely

Relatively high levels of antitoxin can be induced readily in the majority of infants by 2 or 3 injections of toxoid This antitoxin persists for a variable period depending on factors in the child which cannot yet be defined Duration of the immunity may be only a few months but in most instances it will be a few years In the absence of further artificial stimulus to immunity natural factors again become operative and the immunity may be expected to continue if diphtheria bacilli are abundantly present in the environment otherwise to disappear Hence the more successful the campaign to stamp out diphtheria the more certain it becomes that a susceptible adult population will develop if one depends on immunization only during infancy Clearly it is imperative to add to the immunization program two or more recall or boost r injections in later years in order to avoid future difficulty

The question is rai ed occasionally as to whether the diphtheria bacilli gradually lose virulence in the course of convalescence and through mutation or in some other manner become transformed into harmless saprophytes From time to time attempts have been made to approach this possibility experimentally and a certain amount of evidence not altogether convincing in character has been found to support such a view Certainly one cannot anticipate the occurrence of such losses of virulence with regularity for there are many instance of known infection resulting from exposure to released convalescents

The importance of recall injections of toxoid was clearly demonstrated during World War II Prior to the war mass immunization against diphtheria had not been introduced into Germany and the carrier rate remained high In Holland on the other hand artificial immunization of infants had been practiced on a large scale for a number of year Within a year following the German occupation the incidence of diphtheria among the Dutch population as a whole rose 80 fold despite the fact that the diphtheria rate in children under 4 years of age continued to decline A similar increased age incidence of diphtheria has occurred in all other countries where mass immunization of children has been introduced

It is evident from the above discussion that within a population in which diphtheria is prevalent it is incorrect to think of the existence of two simple groups the susceptibl and the immune since the one merges into the other through an infinite series of gradations Those with no previous contact will constitute one fairly sharp group of high susceptibility whose blood upon examination would show no antitoxin Exactly the sam laboratory findings would be manifested by others who e previous contacts with the diphtheria though definite were minimal and by others with more extensive contact who because of lapse of time or personal idiosyncrasy had either lost or failed to produc antitoxin The ma ority of individuals would possess measurable amounts of antitoxin i e more than 0 001 unit per cc and extending upward possibly to many units esp cially if artificial immunization had been employed Clearly then the result of exposure of an individual to infection within such a group would dep nd upon a balance among several factors including at least the virulence of the

organism the degree of exposure (size of the dose), and the unique immune status of that individual To these should perhaps be added other less well defined predisposing factors' such as the general well being of the individual the kind of normal throat flora present and others (see also Dudley et al, 1934)

During recent years outbreaks of diphtheria have occurred in Denmark and Great Britain among immunized populations The studies of Ipsen (1946) and of Edward and Allison (1951) demonstrated that on the first or second day of disease a number of cases showed serum antitoxin levels well above 0 01 to 0 03 units per ml This level has generally been assumed to give complete protection from the toxic manifestations of the infection The disease in immunized persons is usually mild and differs clinically from diphtheria in the completely susceptible individual in that there *is less edema associated with the local lesion* other effects attributable to toxin such as myocarditis and late paralysis do not occur Serum taken several weeks after recovery showed in almost every instance a striking increase in antitoxin titer Edward and Allison reported that nontoxigenic strains of *C diphtheriae* are capable of causing a similar disease but that in this case no rise in serum antitoxin level occurs Similar observations have been made in this country by Frobisher et al (1947)

The Schick Test

In 1913 Schick described a test based on the fact that when a minute amount of diphtheria toxin is introduced intradermally it exerts a local destructive or necrotic effect upon the cells of the skin and the underlying tissue If the blood passing through the tissue contains antitoxin *it will neutralize the toxin* and no injury will occur otherwise a visible reaction develops over a period of days the extent and the severity of which parallel the amount of toxin injected Some individuals (usually immune) may show delayed inflammatory reactions to diphtheria toxin itself to other materials present in toxic filtrates or to peptones used to stabilize the diluted toxin Because these allergic reactions may be confused with reactions due to the primary toxicity of the toxic protein it is customary to include a control in carrying out the Schick test Until recently, this control consisted of crude toxin heated to 60° C for 15 minutes a procedure said to destroy the toxic component without affecting the immunologic reactivity of other proteins concerned in producing the the allergic reaction Since it has been shown that even highly purified toxin is capable of eliciting allergic reactions in certain immune individuals (Pappenheimer and Lawrence, 1948) *toxoid would seem to be more suitable for use as a control than heated toxin* The following Schick test materials have been used in recent years by the Massasetts Department of Health

1 Highly purified diphtheria toxin so diluted in buffered human serum albumin that 1/50 M L D is contained in 0 1 ml of the solution

2 Highly purified diphtheria toxoid diluted in the same dilutent so as to contain 0 01 Lf toxoid in *0 1 ml*

By using purified materials in the Schick test the number of persons showing allergic reactions is reduced and restricted almost entirely to the immune fraction of the population Because both toxin and toxoid in dilute solution are highly sensitive to surface denaturation it is necessary to add a stabilizing agent to the diluent Human serum albumin is now used for this purpose since the commercial peptones employed *in the past as* stabilizing agents not infrequently gave rise to severe local and even general allergic reactions in certain sensitive individuals

The test is carried out by a careful intradermal injection of exactly 0 1 ml Schick toxin into the flexor surface of the forearm A similar injection of the toxoid control is made in the opposite forearm The injected areas should be inspected at 24 or 48 hours *and again between the 4th and the 7th days* The following types of reaction can be distinguished

Positive At the site of toxin injection an area of redness begins to appear at about 24 hours and becomes progressively more pronounced until it reaches a maximum in about a week *At this time it covers an area* up to 3 cm or somewhat more in diameter and may show moderate swelling and slight tenderness There is usually a smaller more deeply colored central area 1 0 1 5 cm in diameter dark red in color which a few days

later turns brown and eventually desquamates sometimes leaving a slightly pigmented surface which may persist for some time The control arm remains completely negative throughout Such a positive test indicates very little or no circulating antitoxin and probable susceptibility to diphtheria

Negative Both arms remain without reaction of any sort Antitoxin is present in reasonable amount sufficient to supply immunity to an ordinary exposure to diphtheria Observations indicate that this level is between $\frac{1}{50}$ and $\frac{1}{100}$ of a unit per cc

Pseudoreaction Inflammatory reactions appear within 12 to 18 hours at the sites of injection of both Schick toxin and control The reactions reach a maximum within 48 hours (occasionally as late as 72 hours) and then fade in contrast with a true positive Schick reaction which persists for many days Individuals who show pseudoreactions are almost invariably immune to diphtheria but are hypersensitive to toxin (and toxoid) or other associated substances present in the solution Almost without exception pseudoreactors show a booster type antitoxin response due to the antigenic stimulation of the Schick test itself

Combined Reaction Commencing like the pseudoreaction delayed reactions develop on both arms After the allergic inflammation has susided the reaction at the toxin site persists as a positive reaction True combined reactions are seldom observed when purified Schick materials are used in the test They indicate a delayed sensitivity to either toxin or protein impurities present in the preparations and that circulating antitoxin is either absent or present in very low titer Such individuals almost invariably respond to the Schick test by antitoxin formation and prove to be negative or pseudoreactors on retest Combined reactions are observed more frequently when commercial peptones are present in the diluent and when crude culture filtrates are used to prepare the test materials

The pseudoreactions observed in the Schick test are of the delayed tuberculin type of inflammatory reaction and are probably the result of previous infections usually inapparent with the diphtheria bacillus Delayed reactions to Schick test materials are seldom observed in young children their frequency increases with age and is highest among populations that have not been actively immunized and where diphtheria is prevalent

Artificial Immunization

When diphtheria toxin is treated with dilute formalin under suitable conditions its toxicity is lost The detoxified protein is called *toxoid* Toxoid retains all of the immunologic specificity of toxin its injection into animals and man gives rise to the formation of diphtheria antitoxin Immunization of children against diphtheria with highly purified toxoid has now become almo t universal practice in this country The purified toxoid is usually injected as an alum precipitate or adsorbed on aluminum phosphate gel as adjuvant Purified toxoid combined with tetanus toxoid and pertussis vaccine is commonly administered to infants and young children

Toxoid is prepared by treating a sterile toxic filtrate of the diphtheria bacillus with 0 3 to 0 5 per cent formalin The formalinized toxin is incubated at 37 C and maintained at slightly alkaline pH (ca pH8) until all traces of toxicity have disappeared Then the toxoid protein is purified by alcohol fractionation at low temperatures or by ammonium sulfate fractionation followed by dialysis The final product either alum precipitated or adsorbed on aluminum phosphate gel should be diluted so as to contain 10 to 20 Lf per immunizing dose of 0 5 or 1 0 ml and 1 10 000 merthiolate is added as preservative Two or 3 doses given 1 month apart are usually adequate for primary immunization followed by booster doses 1 year later

Control of Diphtheria

Information which should make it possible to reduce diphtheria to a minimum within a community is now available Active immunization of infants with diphtheria toxoid combined with tetanus toxoid and pertussis vaccine should be done at the age of a few months It is well to point out that the traditional passive immunity of the newborn child which lasts for a few months can occur only if the mother s plasma contains circulating antitoxin In many communities for reasons discussed above the proportion of immune mothers is steadily decreasing The work of Osborn et al (1952) has demonstrated

that even premature infants are capable of responding an immunizing dose of toxoid given during the first week after birth, although the antitoxin response improves significantly during the first 2 months of neonatal life

A first 'booster' dose of triple antigen should be administered 1 year after the primary immunization and a second 'booster' dose of diphtheria and tetanus toxoid combined should be given at school age These immunizations should be universal and no Schick testing need be done They should result in a reasonably immune population up to adolescence In older children and adults the situation becomes more complex because hypersensitivity reactions to diphtheria toxoid and/or other diphtherial proteins occur in an increasing proportion of the population The frequency of undesirable reactions to diphtheria toxoid increases with age and is greatest in those communities where diphtheria is prevalent and the carrier rate is high While reactions to toxoid among older children and adults are probably never fatal, they may be sufficiently intense to prevent any wholesale immunization of the adult population The reactions are usually of the delayed 'tuberculin type' and may vary in severity from local tenderness and swelling at the injection site to severe generalized illness with fever and complete incapacitation lasting for several days (Pappenheimer et al 1950) Reactions of the immediate anaphylactic type are extremely rare although 1 or 2 such cases have been reported in the literature (Kuhns and Pappenheimer 1952)

It is probably best to handle immunization of adults with diphtheria toxoid on an individual basis wherever possible and to carry out preliminary screening by means of the Schick test Individuals reacting to the Schick control toxoid should not be immunized further Experience has shown that such individuals respond to the test itself with a rapid and relatively high *antitoxin response* In large adult groups such as in military personnel where Schick testing is not feasible the current *practice in this* country and in Canada is to immunize with tetanus toxoid containing a small amount of diphtheria toxoid (*0 5 to 1 Lf* per immunizing dose) The small amount of diphtheria toxoid is in sufficient to produce severe reactions except in a very few highly sensitive individuals but is sufficient to induce *antitoxin production in* all but a minor proportion of the population

Diphtheria Antitoxin

Treatment of diphtheria with antitoxin is still the only specific therapy available Antitoxin is generally produced by immunization of horses with toxoid incorporated in adjuvant Once a horse has developed a sufficiently high antitoxin titer (generally 1,000 units or more per ml serum) bleedings are taken and the antitoxic plasma or serum is fractionated with ammonium sulfate and by dialysis to remove serum albumin and water insoluble globulins Horse antitoxin is generally found in the fast moving gamma 1 (or T fraction) of the water soluble or pseudo globulin fraction Further purification and at least partial despeciation is achieved by digestion with pepsin (Parfentjev 1934 Pope 1939) The peptic digestion not only causes a decrease in species specificity (*Levine et al* 1952) but also causes a splitting in half of the antitoxin molecule (Peterman and Pappenheimer, *1941*)

Assay of Diphtheria Toxin Toxoid and Antitoxin

Precise methods for assay of diphtheria toxin and antitoxin were worked out initially by Paul Ehrlich They furnished a basis for development of general immunochemical methods for assay of biologically active antigens *and their corresponding antibodies* in terms of relative combining units Within certain limits and with certain qualifications (Maaloe and Jerne 1952) the specific activities of both pure diphtheria toxin and antitoxin have been determined so that relative units can now be translated directly into absolute quantities

Ehrlich early recognized the advantage of using antitoxin rather than toxin as a reference standard because of the former s greater stability By assigning arbitrary units to his reference standard Ehrlich was able to measure the unit potency of other antitoxins by determining *their capacity to neutralize* a given amount of toxin relative to the standard In 1922 an international standard diphtheria antitoxin was set up and has been maintained at the State Serum Institute in

Copenhagen Denmark which periodically distributes samples to control laboratories throughout the world for checking their own standards

A few of the methods for estimating the potency of an unknown antitoxin are outlined briefly below By using antitoxins of known unitage the same methods can be modified for determining the specific combining power of diphtheria toxin preparations

1 By definition one unit of antitoxin when mixed with a quantity of diphtheria toxin called an L + dose (L = limes or threshold) and injected subcutaneously into a 250-Gm guinea pig will cause death of the animal within 4 to 5 days The L + dose of a given toxin is determined against a standard antitoxin and then is mixed with dilutions of the unknown serum and injected into guinea pigs

2 The ability of the unknown serum to neutralize a given toxin preparation may be compared with that of an antitoxin of known potency by titration in the skin of rabbits or guinea pigs (see Fraser 1931 for details) Provided that one is dealing with antitoxins of high avidity (Maaloe and Jerne 1952) the antitoxin content of an unknown serum can be estimated with an accuracy of ± 10 per cent or better by rabbit intracutaneous test The method is one of the most sensitive known for determining antibody and as little as 0 001 unit (Ca 0 01 μg antitoxin protein) can be detected

3 The Ramon flocculation reaction provides an in vitro method for determining the potency of an unknown serum (see Chap 6) provided that sufficient floculating antitoxin is present This method is essentially an optimal proportions precipitin test It has been found that when increasing amounts of antitoxin are added to a constant amount of toxin the most rapidly flocculating mixture usually contains an amount of antitoxin just sufficient to combine with and neutralize the toxin When a flocculating antitoxin of known unitage is employed the method can also be used to determine the combining power of either toxin or toxoid in Lf units The flocculation reaction although accurate is considerably less sensitive than the in vivo methods By determining the amount of specifically precipitable protein in the Ramon flocculation test and from the specific activities of highly purified toxin and toxoid the amount of specifically active protein contained in the various units can be estimated and are summarized in the following table

TABLE 26

UNIT OF ACTIVITY	APPROXIMATE MICROGRAMS SPECIFIC PROTEIN PER UNIT
1 unit antitoxin (horse)	10
1 unit antitoxin (horse pepsin treated)	6
1 unit antitoxin (human)	15
1 Lf toxin or toxoid	2 5
1 Minimum lethal dose (M L D) toxin	≤ 0 07
1 Schick test dose toxin	≤ 0 0014

OTHER CORYNEBACTERIA

The corynebacteria are widely distributed in nature and the natural habitat of many strains appears to be the soil While strains other than *C diphtheriae* itself have rarely been implicated in human disease a number of them may cause disease in animals Of the latter *C ovis* is perhaps the most interesting This strain has been shown to cause severe suppurative lesions in sheep horses and cattle a disease known as pseudotuberculosis Morphologically *C ovis* is a pleomorphic bacillus with metachromatic staining resembling the diphtheria bacillus Colonies are frequently yellow in color Carne (1941) has shown that *C ovis* produces a potent toxin that antigenically distinct from diphtheria toxin

For the bacteriologist concerned primarily with human disease corynebacteria other than *C diphtheriae* are important chiefly to the extent that they may confuse the diagnosis of diphtheria

C HOFMANNI

This organism is often found in the throat either healthy or diseased where it lives as a harmless saprophyte It grows readily on the usual laboratory media including Loeffler s medium and tellurite agar Because of the frequency with which it occurs it is the most troublesome of the diphtheroids in connection with the diagnosis of diphtheria As seen in stained smears the Hofmann bacillus is usually somewhat shorter and more plump than *C diphtheriae* and exhibits a tendency to bipolar staining without the appearance of metachromatic granules The trained observer

that even premature infants are capable of responding an immunizing dose of toxoid given during the first week after birth, although the antitoxin response improves significantly during the first 2 months of neonatal life

A first "booster" dose of triple antigen should be administered 1 year after the primary immunization and a second "booster" dose of diphtheria and tetanus toxoid combined should be given at school age These immunizations should be universal and no Schick testing need be done They should result in a reasonably immune population up to adolescence In older children and adults the situation becomes more complex because hypersensitivity reactions to diphtheria toxoid and/or other diphtherial proteins occur in an increasing proportion of the population The frequency of undesirable reactions to diphtheria toxoid increases with age and is greatest in those communities where diphtheria is prevalent and the carrier rate is high While reactions to toxoid among older children and adults are probably never fatal they may be sufficiently intense to prevent any wholesale immunization of the adult population The reactions are usually of the delayed tuberculin type and may vary in severity from local tenderness and swelling at the injection site to severe generalized illness with fever and complete incapacitation lasting for several days (Pappenheimer et al 1950) Reactions of the immediate anaphylactic type are extremely rare although 1 or 2 such cases have been reported in the literature (Kuhns and Pappenheimer 1952)

It is probably best to handle immunization of adults with diphtheria toxoid on an individual basis wherever possible and to carry out preliminary screening by means of the Schick test Individuals reacting to the Schick control toxoid should not be immunized further Experience has shown that such individuals respond to the test itself with a rapid and relatively high antitoxin response In large adult groups such as in military personnel where Schick testing is not feasible the current practice in this country and in Canada is to immunize with tetanus toxoid containing a small amount of diphtheria toxoid (0 5 to 1 Lf per immunizing dose) The small amount of diphtheria toxoid is insufficient to produce severe reactions except in a very few highly sensitive individuals but is sufficient to induce antitoxin production in all but a minor proportion of the population

Diphtheria Antitoxin

Treatment of diphtheria with antitoxin is still the only specific therapy available Antitoxin is generally produced by immunization of horses with toxoid incorporated in adjuvant Once a horse has developed a sufficiently high antitoxin titer (generally 1 000 units or more per ml serum) bleedings are taken, and the antitoxic plasma or serum is fractionated with ammonium sulfate and by dialysis to remove serum albumin and water insoluble globulins Horse antitoxin is generally found in the fast moving gamma 1 (or T fraction) of the water soluble or pseudo globulin fraction Further purification and at least partial despeciation is achieved by digestion with pepsin (Parfentjev 1934 Pope 1939) The peptic digestion not only causes a decrease in species specificity (Levine et al 1952) but also causes a splitting in half of the antitoxin molecule (Peterman and Pappenheimer 1941)

Assay of Diphtheria Toxin, Toxoid and Antitoxin

Precise methods for assay of diphtheria toxin and antitoxin were worked out initially by Paul Ehrlich They furnished a basis for development of general immunochemical methods for assay of biologically active antigens and their corresponding antibodies in terms of relative combining units Within certain limits and with certain qualifications (Maaloe and Jerne, 1952) the specific activities of both pure diphtheria toxin and antitoxin have been determined so that relative units can now be translated directly into absolute quantities

Ehrlich early recognized the advantage of using antitoxin rather than toxin as a reference standard because of the former s greater stability By assigning arbitrary units to his reference standard Ehrlich was able to measure the unit potency of other antitoxins by determining their capacity to neutralize a given amount of toxin relative to the standard In 1922 an international standard diphtheria antitoxin was set up and has been maintained at the State Serum Institute in

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usually recognizes it without difficulty, but assurances comes only with extensive experience On the tellurite plate, colonies of *C Hofmanni* may resemble closely those of *mitis* strains of the diphtheria bacillus Here also experience aids in differentiation, the saprophyte often forming a colony with a rather wide grayish or white margin and a dark central portion, whereas the pathogen tends to produce a more uniformly dark or black colony The modification of Lev (1947) permitting the detection of glucose fermenting colonies of tellurite agar should be valuable Since there are known to be various serologic types of Hofmann as well as atypical varieties of the diphtheria bacillus it follows that complete certainty must depend upon the isolation of the strain and examination of its fermentative properties and its toxigenicity *C Hofmanni* fails to ferment glucose and of course lacks the ability to form toxin

C XEROSIS

Isolated originally from cases of xerosis conjunctivae this organism was shortly found to be present in many normal conjunctivae and probably is without pathologic significance It is occasionally present in the throat though much less commonly than the Hofmann bacillus Morphologically it is probably indistinguishable from *C diphtheriae* Its growth on artificial media tends to be less vigorous than that of the diphtheria bacillus and the colonies on tellurite plates are somewhat smaller than those of either the *mitis* or the *gravis* strains but larger than those of *intermedius* diphtheria Here again in case of doubt one must resort to isolation fermentation and virulence tests

For a description of other varieties of corynebacteria from both human and animal sources, the reader should consult one of the larger handbooks of medical and veterinary bacteriology

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due the greatest part of our knowledge of the immunologic and chemical properties of these capsular constituents It has been convincingly shown that the production of the capsular polysaccharides is essential to the pathogenicity of pneumococcus and that the antibodies which protect man or animals against infection are directed against this capsular material

Many of the biologic properties of pneumococcus are similar to those of various species of streptococci especially streptococci of the viridans group It is for this reason that certain taxonomists have included pneumococcus as a species of streptococcus under the name *Streptococcus pneumoniae* though agreement on this designation is not universal The most important properties which distinguish pneumococcus from the green producing streptococci are its predilection in causing pneumonia its virulence for laboratory animals on primary isolation and its dissolution by bile salts and other surface active agents

MORPHOLOGY

In the sputum the pus and the lungs of patients with pneumonia pneumococcus appears singly in pairs or in short chains of ovoid or lanceolate cocci When in pairs the adjacent ends of the cocci are generally rounded with the distal ends pointed The appearance in artificial culture medium is similar except that short chains of cocci are seen more commonly especially in young cultures During the active phases of growth pneumococcus is gram positive but as the culture begins to age gram negative forms appear which retain their coccoid shape On continued incubation the gram positive forms gradually disappear and eventually are replaced entirely by gram negative cocci This is followed by further autolytic changes so that finally no formed elements but only gram negative debris can be seen

The process of autolysis can be greatly enhanced by surface active compounds Upon the addition of the whole bile or bile salts such as sodium desoxycholate or taurocholate pneumococcus autolyzes with great rapidity clearing of a turbid suspension of organisms occurring within a matter of minutes The phenomenon of bile solubility is due to activation of the autolytic enzymes of pneumococcus and if the enzymes are first inactivated by heating the suspension of cocci at 65° C for 30 minutes autolysis no longer takes place either spontaneously or when bile is added The mechanism of activation of the autolytic enzyme system by surface active substances has not been explained though it seems not unlikely that activation results from alteration or removal from the cells of a normal inhibitor of autolysis

On the surface of solid media such as fresh peptone infusion agar plates to which blood has been added young cultures of the virulent organisms form smooth glistening unpigmented dome shaped colonies which are circular in outline and in general vary from 0 5 to 1 5 mm in diameter Colonies formed by Type III pneumococcus are larger and more mucoid than those produced by other types and commonly attain a diameter of from 2 to 3 mm on blood agar The greater size and more mucoid consistency of the Type III colonies is due to the larger amount of capsular polysaccharide synthesized by this type As the cultures on blood agar age autolytic changes appear The centers of the colonies collapse often leaving a small central papilla with a depressed area intervening between it and the raised outer margin of the colony

Surrounding the colonies on blood agar and becoming more apparent with continued incubation at 37 C there is a zone of alpha hemolysis showing the characteristic greenish brown color

In fluid media encapsulated pneumococci grow diffusely and tend to sediment only when the pH has fallen because of acid production This occurs in media containing relatively large amounts of glucose or other fermentable carbohydrates

NUTRITION

In the older literature pneumococcus is characterized as a fastidious micro organism indicating difficulties in cultivation and maintenance in the viable state These difficulties were probably multiple in nature and due not only to partial deficiencies in essential nutrients but also to improper oxidation reduction potential of the medium

The importance of the oxidation reduction potential has been clarified especially through the studies of Dubos (1929) When peptone

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The Pneumococci

Gram positive coccus frequently of lancet shape, usually are arranged in pairs or short chains and possess an easily demonstrable capsule Pneumococcus is nonmotile and does not form spores It is lysed by bile salts and other surface active agents It is classified into types on the basis of immunologic and chemical differences in the highly polymerized polysaccharides which compose its capsule It is a normal inhabitant of the upper respiratory tract of man and certain animals and causes infection primarily in the respiratory tract and adjacent structures especially pneumonia sinusitis otitis conjunctivitis and meningitis

Synonyms *Diplococcus pneumoniae, Streptococcus pneumoniae*

HISTORY

Pneumococcus was first isolated and cultured in 1881 by Pasteur in France and independently in the same year by Sternberg in New York In both instances saliva of persons who were not suffering from a respiratory disease was injected into rabbits from whose blood the organisms were subsequently isolated Neither Pasteur nor Sternberg appreciated its relationship to disease This was demonstrated by the independent studies of Frankel and of Weichselbaum between 1884 and 1886 who showed pneumococcus to be the most frequent cau e of lobar pneumonia in man

Classification of pneumococcus into types began with the work of Neufeld and Handel in Germany in 1909 and 1910 with the observation that strains of pneumococci differ in their immunologic properties Shortly afterward Dochez and Gillespie (1913) in New York subdivided pneumococci into 3 distinct types and a 4th heterogeneous group on the basis of agglutination reactions and protection tests in mice Simultaneously Lister in South Africa reported similar findings The great significance in the observations of Dochez and Gillespie lies in the fact that 2 of the types described by them Types I and II, have been more commonly associated with pneumococcal pneumonia in adults than any of the other types and together have been found to be responsible for about one half of all cases during the 20 year period between 1920 and 1940 when typing was commonly carried out Type III the 3rd type described by them is more commonly carried in the normal human pharynx than any other single type of pneumococcus and is likewise a frequent cause of pneumonia and other lesions Subsequent study of the heterogeneous group IV of Dochez and Gillespie has resulted in the recognition of more than 75 distinct pneumococcal types

The basis for the immunologic differentiation of pneumococci into types was demonstrated by Dochez and Avery (1917) to reside in the elaboration during growth of so called specific soluble substances (SSS) which constitute the capsules of the micro organisms Heidelberger and Avery (1923) showed that the specific soluble substances are carbohydrate in nature and to them and Goebel is

meat infusion broth is exposed to the air the medium becomes oxidized Under such circumstances large inocula of pneumococcus must be used to obtain growth The large inoculum is able through its metabolic activity to lower the redox potential sufficiently to permit growth whereas with a small inoculum this may not occur When the medium is reduced by placing it under reduced pressure by heating to drive off dissolved oxygen or better by addition of a reducing agent such as cysteine or thioglycollic acid growth can be initiated from a very small inoculum Media satisfactory for pneumococcal growth can be prepared readily from fresh meat infusion with the addition of any good brand of peptone The pH of the medium should be between 7 2 and 7 4 after sterilization which should be accomplished with a minimum of heating Media made with a fresh meat infusion base are in general more satisfactory than the dehydrated media which are commercially available

The optimum pH for growth of pneumococcus is stated to be 7 8 Excellent growth can be obtained over the range of pH 7 2 to 7 8 but from the practical viewpoint it is probably desirable to use media of pH 7 2 to 7 4 because there is less chance of deleterious alterations occurring on sterilization by heat at lower pH values

All of the nutritional factors required for growth of pneumococcus have not been determined However a partially defined medium has been prepared by Adams and Roe (1945) which supports the growth of many but not all strains This medium is basically that designed by Bernheimer et al (1942) for the cultivation of Group A streptococci with the addition of asparagine and choline which have been shown to be essential for growth of most pneumococcal strains tested In addition to an acid hydrolysate of casein supplemented by l cystine and l tryptophane the medium contains the following accessory growth factors biotin nicotinic acid pantothenic acid choline pyridoxine thiamine riboflavin adenine and uracil Of these the first 4 are known to be essential In common with Group A streptococci pneumococcus has been found to require large amounts of glutamine Immediately before inoculation sodium bicarbonate is added to provide CO_2 which is essential for initiation of growth and th redox potential is lowered by addition of a reducing agent such as thioglycollic acid Glucose is used as carbon source since pneumococcus derives almost all of its energy requirements from anaerobic glycolysis Table 27 which is taken from the paper by Adams and Roe lists the components of the partially defined medium and the method of preparation Further work is required to find out if all of the accessory growth factors listed are essential for pneumococcal growth and to determine the amino acid requirements

Defined media such as that described are especially useful for chemical investigations of bacteria for example the preparation of capsular polysaccharides or somatic proteins because all of the constituents of the medium are dialyzable and can be eliminated by this means

For routine cultivation of pneumococcus it is preferable to use complex media such as peptone fresh meat infusion broth to which blood has been added Growth of all strains can be obtained autolysis is less rapid than in the defined medium described and cultures can be stored in the icebox for prolonged periods of time The presence of blood is particularly important for storing cultures Presumably this is because the catalase present in the red cells destroys hydrogen peroxide produced by pneumococcus Pneumococcus contains neither catalase nor peroxidase and in consequence H_2O_2 accumulates in its environment if air is present in amount probably great enough to affect viability of the organisms In any case although blood does not improve the growth of pneumococcus when added to a good medium its presence favors preservation of viability of the organisms on storage

PHYSIOLOGY

As noted above pneumococcus derives most *of its energy requirements from the fermentation* of glucose Practically all of the glucose metabolized can be accounted for by the lactic acid which accumulates in the medium during growth The metabolism of pneumococcus is thus essentially anaerobic However it can grow in the presence or the absence of oxygen and hence has been classified as aerobic or facultatively anaerobic Growth is somewhat better under anaerobic conditions even though pneumococcus does not possess th cytochrom system or catalase Better growth under aerobic conditions apparently is due to the capacity to take up oxygen through a flavin

TABLE 27 COMPOSITION AND METHOD OF PREPARATION OF A PARTIALLY DEFINED MEDIUM FOR PNEUMOCOCCUS*

Basal Medium—for 1 liter of medium	
Acid hydrolysate of casein	200 ml of 10% solution
l Cystine	150 mg
l Tryptophane	20 mg
KCl	3 Gm
$Na_2HPO_4 \cdot 12H_2O$	7.5 Gm
$MgSO_4 \cdot 7H_2O$	0.5 Gm
Distilled water to make	900 ml

Adjust pH to 7.5 heat to boiling filter and tube in 9 ml amounts or appropriate multiple Autoclave

Solution 1—vitamin mixture for 12.5 liters	
Biotin	0.015 mg
Nicotinic acid	15.0 mg
Pyridoxine	15.0 mg
Calcium pantothenate	60.0 mg
Thiamine	15.0 mg
Riboflavin	7.0 mg
Adenine sulfate	150.0 mg
Uracil	150.0 mg

Dissolve in 100 ml of distilled water and sterilize by filtration Store in refrigerator

Solution 2—salt mixture for 50 liters	
$FeSO_4 \cdot 7H_2O$	50 mg
$CuSO_4 \cdot 5H_2O$	50 mg
$ZnSO_4 \cdot 7H_2O$	50 mg
$MnCl_2 \cdot 4H_2O$	20 mg
HCl concentrated	1 ml

Dissolve in 100 ml of distilled water and sterilize by boiling

Addition mixture per liter of medium	
Vitamin mixture (solution 1)	8.0 ml
Salt mixture (solution 2)	2.0 ml
Glucose (20% solution)	10.0 ml
Glutamine	200 mg
Asparagine	100 mg
Choline	10 mg
$CaCl_2 \cdot 2H_2O$	10 mg
Distilled water to make	50 ml

Sterilize by filtration and store in refrigerator Add 0.5 ml to each 9 ml of basal medium This addition mixture should not be kept longer than a few weeks as the glutamine is unstable Solutions 1 and 2 appear to keep indefinitely

Bicarbonate—thioglycollate mixture	
Thioglycollic acid	10%

Add 1 ml of thioglycollic acid to 9 ml of sterile distilled water mix well and heat in boiling water bath for 10 minutes

Bicarbonate Weigh 200 mg samples of sodium bicarbonate into test tubes and autoclave

Add 10 ml of sterile distilled water to a test tube containing bicarbonate and dissolve the latter Then add 0.2 ml of 10 per cent thioglycollic acid and mix well immediately Add 0.5 ml of the mixture to each 9.5 ml of medium This bicarbonate thioglycollate mixture is unstable and must be made up and added to the medium just prior to inoculation

* From Adams M H and Roe A S 1945 A partially defined medium for cultivation of pneumococcus J Bact 49 401

caused in both cases by the coiling and the piling up of the long coccal chains

It has been customary in the past to speak of anti S serum as causing reversion from S to R thereby inferring that anti S serum in some way depresses capsular synthesis and that anti R serum stimulates it This is an incorrect view Any pneumococcal culture although superficially homogeneous contains a large number of variants and S cultures throw off R mutants at a fairly constant rate It has been shown experimentally that if a mixture of S and R cells is inoculated into broth containing anti S serum R cells have a selective advantage over S and conversely when such a mixture is grown in anti R serum S organisms have a selective advantage over R forms

When certain cultures of R pneumococci are grown in broth containing anti R serum or when the R culture is inoculated into mice the R pneumococci disappear and are replaced by S pneumococci of the same serologic type as that from which the R strain was derived by mutation and selection With some R cultures this does not occur R cultures which revert to the S form either contain S organisms in numbers so small that they are undetectable unless they are cultivated in a selective environment or else back mutation from R to S has occurred

The enhancement of virulence which occurs upon repeated animal passage is due to a similar process of selection The less virulent *forms are* destroyed by the host the more virulent are able to survive and thus are specifically selected Selection of drug resistant variants or mutants by cultivating the organisms in increasing concentrations of a particular drug may also be cited as another example of the same process

Variation in pneumococcus has also been studied by means of *transformation reactions* As shown originally by Griffith (1928) pneumococcus of one specific type can be transformed to another specific type by way of the R variant In his original experiments Griffith obtained R cells from a culture of pneumococcus Typ II by cultivation in Type II antiserum Then the living R cells derived from Type II were subcutaneously inoculated into mice along with large numbers of heat killed Type III S cells Upon death of the mice cultures of the heart blood showed the presence of living Type III S cells In other words R cells derived from a strain that originally synthesized Type II capsular polysaccharide have been transformed so that they now produce the Type III capsule This alteration in polysaccharide synthesis results from a permanent genetic change in the recipient cells Subsequently it was shown by Avery MacLeod and McCarty (1944) that type transformation can be effected by purified preparations of highly polymerized desoxyribonucleic acids (DNA) DNA from S cells of a particular type when applied to R *cells derived from another type cause the* latter to synthesize capsular polysaccharide of the type from which the DNA was prepared Exogenous DNA which has been added to the culture medium under appropriate environmental conditions is taken by the growing cells incorporated into their genetic structure replicated and transmitted to the descendants Therefore transformations in pneumococci have great biologic significance because by these reactions it was demonstrated originally that DNA possesses genetic specificity and a method was provided for studying the characteristics of the genetically active material

It should be borne in mind that DNA as extracted from pneumococci or other bacterial cells does not consist of a single molecular species but rather of a mixture of many desoxyribonucleic acids which presumably differ from one another not only in respect *to the nature and the sequence of structural* units in the nucleotide chains but also in the manner and the extent of polymerization Depolymerization effected by enzymatic chemical or physical means destroys the specificity of DNA as evidenced by the loss of its ability to cause genetic transformation

The mechanisms which enable bacterial cells to take up free DNA from the environment and incorporate it into their genetic constitution is unknown The long threadlike molecules with molecular weights estimated by different investigators to be from 1 000 000 to 16 000 000 are transported across the cell wall and the cytoplasmic membrane and having gained access to the interior of the cell can be incorporated into the nuclear apparatus by a process which appears to be analogous to crossing over in genetic exchanges in higher species

containing enzyme system Under these conditions H_2O_2 is produced by the autoxidation of reduced flavoprotein As previously noted, H_2O_2 accumulates in the medium because pneumococcus possesses neither catalase nor peroxidase

One of the most important factors limiting the growth of pneumococcus is the lowering of the pH of the medium due mainly to the accumulation of lactic acid although with some strains significant amounts of formic and acetic acid may be produced (Friedemann 1938) Massive growth can be obtained by supplying a large amount of glucose and neutralizing the lactic acid with NaOH as it is formed For the preparation of the capsular polysaccharides neutralization with NaOH should be avoided Even though alkali is added cautiously and with very careful stirring of the culture during addition, it has been shown that degradation of the polysaccharides is likely to occur (Heidelberger et al 1950)

IDENTIFICATION

Members of the heterogeneous group of gram positive cocci collectively described under the term viridans streptococci" are the only bacteria which are commonly confused with pneumococcus However a single test may be used to separate them—that is whether they are bile soluble or not All or practically all strains of pneumococcus are bile soluble whereas the viridans streptococci are insoluble This test is very reliable, provided that the conditions are proper and appropriate controls are used In general it is advisable to remove the living organisms by centrifugation from the medium in which they are grown especially if it contains blood or other protein materials The organisms should be suspended in isotonic saline adjusted so that the pH is in the neutral range Under these circumstances pneumococci go into solution with great rapidity either at room temperature or at 37° C upon the addition of an equal volume of 10 per cent bile Dissolution is faster at 37° C, and if the suspension has not cleared within 30 minutes at this temperature it is most unlikely that the micro organism in question is pneumococcus

The fermentation of inulin has been used as a differential test to distinguish pneumococci from streptococci While it is true that inulin fermentation is a property of pneumococcus it is not a reliable test when used by itself since certain streptococci, especially those of the *salivarius* group share this capacity

Another property which is of great use in identifying pneumococcus is its virulence for mice on inoculation Streptococci of the viridans group are avirulent for mice, whereas most strains of pneumococci are highly virulent even on primary isolation However, there are important exceptions since certain commonly encountered pneumococcal types for example, Type XIV are not mouse virulent

VARIATION

On cultivating encapsulated pneumococci in nutrient broth to which antipolysaccharide antibodies have been added after a few serial transfers many of the cocci will be found to be devoid of capsules (Stryker, 1916) For example when pneumococcus Type I is grown in the presence of Type I antipneumococcal serum encapsulated Type I pneumococci disappear and are replaced by nonencapsulated forms With loss of the capsule the organism lose type specificity and virtually all of their pathogenicity

On the surface of an agar medium encapsulated pneumococci form characteristic smooth glistening colonies The pneumococci composing the smooth colonies are referred to as *mucoid smooth* or *S* organisms With disappearance of the encapsulated forms such as occurs on cultivation in homologous antiserum the colonies formed on agar lose their glistening appearance and tend to have a finely granular or roughened surface The organisms composing these colonies are spoken of as *rough* or *R*

A third form characterized by colonies with a markedly roughened surface has been described by Dawson (1934) to appear following prolonged incubation of cultures planted on the surface of agar plates Whether grown in fluid or on solid media the organisms of such strains are arranged in chains that may contain several hundred cocci In strains of *viridans* streptococci where colonies having a very rough surface are commonly seen the cocci also occur in chains of great length The markedly roughened surface seems to be

TABLE 28 PROPERTIES OF THE SPECIFIC CAPSULAR POLYSACCHARIDES OF SIX PNEUMOCOCCAL TYPES

TYPE	[α]	ACID EQUIVALENT	MOLECULAR WEIGHT	N	REDUCING POWER ON HYDROLYSIS AS GLUCOSE	CONSTITUENTS IDENTIFIED	COMMENT
				per cent	per cent		
I[1]	+265 to +277	650	171 000-	4 6	30	Galacturonic acid (307) N acetyl glucosamine acetic acid	
II[3]	+60	1030	504 000	0 2 (none 6b)	95	D glucose D glucuronic acid L rhamnose 1 3 7	Cross reacts with K friedlanderi Type B All glucose in form 1 4 6 branch points (3a) Cros reacts with lung galactan (3c) and gum arabic (3d)
III[4]	−33 to −37	350	141 000	0 05	85	Glucuronic acid glucose 1 1	Type III antiserum cross reacts with Type VIII polysaccharide and vice versa Oxidized cotton cross reacts with Type III and VIII horse sera (4b) Cellobiuronic acid is the structural unit of Type III
VIII	+121	703	140 000	none	87	D glucose D galactose D glucuronic acid 2 1 1	Cellobiuronic acid in common with Type III
XIV[6]	+12 5	—*		2 0	84	D galactose N acetyl D glucosamine D glucose	Type XIV antiserum cros reacts with blood group sub tances e pecially A and with lung galactan (3c)
XVIII[7]	+86 to +89	—**		0 3	58	D gluco e L rhamnose phosphate 5 1 1	

* Contains no acid groups

* Contains phosphate bound in secondary linkage

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Similar genetic exchanges occur in various *Enterobacteriaceae* by means of transduction and through the phenomenon of recombina tion Both of these processes differ from trans formation in the mechanisms by which genetic material is transferred In transduction trans fer is effected by lysogenic bacteriophages, whereas in recombination the cells conjugate with formation of partial zygotes

The usefulness of transformation reactions in genetic studies has been greatly extended by the discovery of additional hereditary char acteristics of pneumococci and other bacterial species which can be altered by them The demonstration by Hotchkiss (1951) that drug resistance can be transferred to sensitive cells by DNA from resistant mutants has provided a series of genetic markers that permit quan titative studies to be carried out with relative ease (Hotchkiss 1957) Other pneumococcal transformations include alterations in the quantity of SSS produced changes in the serologic type of M proteins capacity to pro duce an adaptive enzyme, mannitol phosphate dehydrogenase changes in R colonial mor phology Recently it has been demonstrated (Bracco et al 1957) using streptomycin re sistance and resistance to optochin (ethyl hydrocupreine) as genetic markers that trans formations mediated by the respective DNA s can take place reciprocally between pneumo-cocci and distantly related streptococci

ANTIGENIC STRUCTURE

Pneumococcus can be divided into 75 or more types on the basis of differences in the capsules which surround the cells The cap sules are composed of highly polymerized polysaccharides which are immunologically distinct for each type Extensive studies car ried out especially by Heidelberger Goebel Avery and by Stacey and his associates have provided a chemical basis for the immunologic specificity of the capsular polysaccharides of a number of types Table 28 shows certain of the chemical properties of polysaccharides of Types I II III VIII XIV and XVIII

The isolated purified capsular polysaccha rides of pneumococci are antigenic for some species such as man and mouse but not for the rabbit, although the serum of rabbits im munized by the injection of intact pneumo coccal cells contains antibodies which react specifically with the isolated capsular poly saccharides of the same type That is to say the isolated polysaccharides are haptenes for the rabbit but antigens for man and mouse However it has been demonstrated recently that rabbits which have *undergone primary* immunization with intact pneumococci uni formly show repeated secondary or anamnestic responses upon injection of purified polysac charides of the homologous type The mech anism by which a compound that is haptenic in normal animals becomes antigenic in pre viously immunized animals is unknown

The somatic portion of the cells, although antigenically similar in all types is not identi cal Antisera prepared against R pneumococci derived from one type agglutinate homologous R organisms to higher titer than heterolo gous R cells This is due in part to the pres ence of prolaminelike M proteins which are antigenically dissimilar in pneumococci of different capsular types and in the R organ isms derived therefrom (Austrian and Mac Leod 1949) In nature, cells of a particular capsular type commonly possess an M protein characteristic for that type However, cap sular polysaccharide and M protein can vary independently of one another as shown par ticularly by transformation reactions Pneu mococcal M proteins have chemical properties similar to M proteins of Group A streptococci but are immunologically distinct

Like β hemolytic streptococci the somatic portion of pneumococcus contains a C or cel lular carbohydrate described by Tillett, Goebel and Avery (1930) which is immunologically as characteristic of pneumococcus as a species as are the C carbohydrates of the Lance field groups of streptococci However unlike β hemolytic streptococci the C carbohydrate of pneumococcus has not been used as a means of classifying pneumococci from the syste matic point of view The studies of Goebel and Adams (1943) have shown that the C car bohydrate forms a portion of the Forsmann (heterophile) antigen of pneumococcus

Antibodies to the whole somatic portion of pneumococcal cells or any fractions of it so far studied show slight protective power for experimental animals in contrast with the great protection given by antibodies to th capsular polysaccharides Tillett (1928) dem onstrated that rabbits immunized with heat killed R pneumococci develop resistance to infection with virulent encapsulated pneumo

tant exception is Type XIV which is avirulent for mice despite the fact that it is one of the most frequent causes of lobar pneumonia in children It is probable that if systematic study was made other types which are uniformly avirulent for mice would also be found

Similar observations have been made in the rabbit with pneumococcus Type III which although highly virulent for man and mouse is almost avirulent for the rabbit However an occasional strain of Type III has been described which posse es pathogenic properties for rabbits A satisfactory explanation for the difference in virulence of these strains of pneumococcus Type III has not been found

Both dogs and monkeys have been used extensively for the study of pneumococcal lobar pneumonia produced by experimental inoculation by the intratracheal route and under appropriate conditions both are highly susceptible Felines are relatively insusceptible to experimental infection and birds are especially resistant

It is apparent from the observations on pathogenicity described above that the data obtained from studies of one animal species cannot be transferred to anothe Virulence in the case of pneumococcus should be defined only for the species in which it has been measured and one cannot infer for example that because a strain or type is virulent for the mouse it is comparably virulent for man or vice versa

FACTORS INVOLVED IN PATHOGENICITY OF PNEUMOCOCCUS

Pneumococcus is perhaps the best illustration of a bacterial species that produces disease apparently solely through invasive properties in other words because of the capacity to invade and multiply in living tissues without evidence that soluble toxins in the usual sense play a part *Cl botulinum* at the other end of the scale exerts its pathogenicity entirely through a potent exotoxin which is produced outside of the body and causes disease upon absorption through the gut *Cl botulinum* has no invasive capacity whatsoever Between these two extremes of purely invasive and purely toxigenic pathogens are many species that possess both capacities in varying degrees

The search for a toxin produced by pneumococcus which can account for its disease-producing capacity has been unsuccessful It is true that two toxic principles have been identified but since neither of them is liberated in detectable amount except upon autolysis of the bacterial cells it is doubtful that either plays a significant part in pneumococcal pathogenicity

Pneumolysin an oxygen labile or O hemolysin is liberated from pneumococcus especially on autolysis (Cole 1914) It is related serologically to the O hemolysins produced by hemolytic streptococci *Cl tetani* and *Cl welchii* as shown by Todd (1934) It should be noted however that hemolysis is never a feature of even overwhelming pneumococcal infection and also that in the case of the 3 toxigenic species noted above which produce similar O hemolysins it has not been proved that these substances play a significant part in disease caused by the respective species

Under conditions of autolysis there is also liberated from pneumococcus a purpura producing principle whose effect can be demonstrated both in the skin and the internal organs of experimental animals injected with sterile pneumococcal autolysates Once again however purpura or other hemorrhagic incidents are not seen except in the rarest instances in pneumococcal infection whether in man or other animals so that the purpura producing principle would appear to have little significance in the pathogenicity of pneumococci

So far as present information goes pneumococci produce disease and death solely through their capacity to multiply in the tis ues However it is possible that a toxin is produced in the tissues which differs in nature from any previously studied and that its demonstration requires new methods

Another possible mechanism whereby such a rapidly growing pathogen might produce tissue damage is through the production of local or general acute deficiencies in one or more essential metabolites whether vitamins amino acids purines or constituents of other cell components The demands of bacterial cells having a generation time of from 20 to 30 minutes on available supplies of essential metabolites in the animal body must be great indeed and conceivably the micro organisms might compete for limited supplies of such materials on a more favorable basis than the

cocci of Types I II and III Subsequently, Dubos (1938) extracted a soluble antigen from pneumococcal cells which caused the production in rabbits of antibodies capable of protecting mice against infection with both homologous and heterologous types The nature of the antigen responsible for the broad immunity studied by Tillett and by Dubos is unknown though there is evidence that the C polysaccharide may be involved (Enders Wu and Shaffer 1936) Antibodies to M protein are not protective It is worth emphasizing however that non type specific resistance to infection obtained by the methods described above is slight when compared with the high degree of immunity afforded by type specific antibodies and probably plays a minor part in protection against pneumococcal infection

DISTRIBUTION

Pneumococcus has a world wide distribution It is a normal inhabitant of the nasopharynx of man under all climatic conditions In many parts of the world Types I and II have been reported as the most frequent causes of human disease, although in normal people other types are carried more commonly in the pharynx Among other animal species guinea pigs monkeys and rats are the only ones that are known to harbor pneumococci commonly These species have little or no importance as reservoirs of pneumococci so far as human disease is concerned However epizootics of pneumococcal pneumonia occur among monkeys in captivity and in the guinea pig pneumococcus Type XIX causes one of the most frequent and fatal epizootic diseases of this species None of the other types is of importance in causing disease in guinea pigs, though they may become carriers if other types are experimentally implanted in the nasopharynx In rats severe epizootics have been caused by pneumococcus Type II (Mirick et al 1950)

PATHOGENICITY AND HOST RANGE

Infections in man can be caused by any of the more than 75 serologic types of pneumococcus Of this large number of types however a relatively small number accounts for most human disease Types I II and III cause approximately one half of all the cases of lobar pneumonia in adults in different parts of the world (Heffron 1939) with 8 types causing about 75 to 80 per cent of the total number of infections Table 29 which is taken from the summaries of Heffron, shows the incidence of lobar pneumonia caused by various types in 3 large cities in northeastern United States

TABLE 29 DISTRIBUTION OF PNEUMOCOCCAL TYPES IN ADULTS WITH LOBAR PNEUMONIA

PNEUMOCOCCAL TYPE	NUMBER OF CASES	PER CENT OF TOTAL
I	1 063	28 6
II	425	11 4
III	500	13 5
IV	131	3 5
V	298	8 0
VI	66	1 8
VII	240	6 5
VIII	287	7 7
Total I VIII	3 010	81 0
All other types	703	19 0
	3 713	100 0

It should be noted that the figures given in Table 28 are for cases of pneumonia having anatomically a lobar distribution Pneumococci are also the most frequent causative agents of bronchopneumonia but the accuracy of the figures for this anatomic variety is not so great as for lobar pneumonia

In children below the age of 12 the distribution of pneumococcal types in lobar pneumonia differs from that in adults with Types XIV I VI, V VII and XIX in that order, causing more than half the cases (Heffron) It is of interest that the capsular polysaccharide of Type XIV is immunologically related to blood group A substance (Beeson and Goebel 1939)

Among laboratory animals the mouse is the most susceptible species although rats and rabbits are also highly susceptible to experimental inoculation The virulence of most types for mice can be enhanced quickly by repeated passage due to the selection by this means of the most virulent mutants from a mixed population of cells of variable virulence With many types as few as 1 to 5 cocci inoculated intraperitoneally into mice will cause death within 48 hours However an impor

by mixing R and S pneumococci respectively with heparinized normal human blood Phago cytosis *of the R cells takes place with great* rapidity whereas the leukocytes have a limited capacity to phagocytose the S forms However if a small amount of type specific antiserum is added the S cells are taken up rapidly by the leukocytes The combination of specific anti body with the capsular polysaccharide removes its antiphagocytic property The antiphago cytic action of the capsule appears to be me chanical and not to be caused by a toxic effect on the leukocytes The detailed studies of Wood and his co workers (see review by Wood 1951 52) indicate that when phago cytosis experiments are carried out on smooth surfaces such as glass or wax encapsulated pneumococci are pushed about by the leuko cytes which have difficulty in embracing the organisms within the pseudopodia because the pneumococci slide away over the smooth sur face On the other hand on a roughened surface such as blotting paper filter paper fibrin films or fixed sections of lung the leuko cytes take up encapsulated pneumococci fairly readily even in the absence of specific anti body because the bacteria can be pinned against obstructions on the roughened surface and then enclosed in the pseudopodic exten sions Intracellular digestion ensues Wood has shown that the nature of the surface influences phagocytosis in vivo also

If antibody is added to the mixture of en capsulated pneumococci and phagocytes the nature of the surface on which the experiment is carried out makes little difference The com bination of antibody with the capsule appears to make the pneumococci sticky since now they adhere to the leukocytes and are readily ingested

From the foregoing observations it should be apparent that the presence of type specific antibody is not a prerequisite for phagocytosis of encapsulated pneumococci In the absence of antibody phagocytosis is relatively slow but it is enhanced enormously when antibody is added

The studies of Dubos and Avery on an enzyme which specifically digests the capsular polysaccharide of pneumococcus Type III both when the polysaccharide is in the iso lated purified state and when it is present on the surface of the living virulent cells pro vide further evidence for the importance of the capsule in pathogenicity of pneumococci (Dubos 1939 40)

From the soil of a cranberry bog an aerobic sporulating bacillus was isolated which when grown *in a medium containing the polysac* charide of pneumococcus Type III as the sole carbon source elaborates a specific adaptive enzyme having the capacity to hydrolyze the capsular polysaccharide of pneumococcus Type III but none of the other pneumococcal polysaccharides In the test tube the Type III enzyme digests and removes the capsule from either dead or living Type III pneumococci moreover when injected into mice rabbits or monkeys infected experimentally with pneu mococcus Type III as long as 18 hours after infection the enzyme prevents the death of the animal The sole action of the SIII en zyme is to remove the pneumococcal capsule by digesting it with the result that the decap sulated bacteria become highly susceptible to phagocytosis It should be emphasized that digestion of the Type III capsule from the living organisms in vitro in no way affects their viability although as long as active en zyme is present the capsular material is digested as rapidly as it is produced How ever when transferred to fresh medium lack ing the SIII enzyme the pneumococci again produce their capsules normally

A considerable body of evidence has been accumulated therefore which emphasizes the importance of the pneumococcal capsule in pathogenicity The part played by somatic factors on the other hand has received little attention though there is evidence of their significance One of the most clear cut illus trations of the influence of somatic factors is in the case of two well known laboratory strains of pneumococcus Type III known as A66 and SV III Strain SV III is highly viru lent for rabbits whereas A66 has very little virulence even upon repeated animal passage The capsular polysaccharide is identical in both strains Moreover if the capsule is switched from A66 to SV III and vice versa by means of transformation reactions no al teration in virulence occurs (Shaffer Enders and Wu 1936) In other words the differences in virulence of these strains appear to depend on the somatic portion of the cells

Pneumococcus may be avirulent for a spe cies because of the nature of the capsule Type XIV for example is avirulent for the mouse on this basis R pneumococci derived

cells of the host with a resulting acute deficiency for the host cells Other means by which a primarily invasive organism such as pneumococcus causes tissue damage might be suggested but would serve only to emphasize our state of ignorance

Whatever may be the ultimate means by *which pneumococcus produces damage to tissues* a fact of great significance is that most of the pathogenic power of this species is exerted through possession of a nontoxic surface component—the pneumococcal capsule All virulent strains of pneumococcus possess a capsule which forms a protective mantle about the cells The sole function of the pneumococcal capsule appears to be to delay or prevent ingestion of pneumococci by the phagocytic cells of the body *The free isolated* polysaccharides which compose the capsules in the different types are nontoxic upon injection in even very large doses Evidence of the role of the capsule in pathogenicity has been obtained in a variety of ways a number of which will be described

Upon immunization of animals by injecting them with killed encapsulated pneumococci antibodies are formed which are able to protect other animals infected experimentally with the same type For example a serum prepared by immunizing rabbits with pneumococcus Type I protects against experimental infection with living Type I pneumococci but will not protect against infection with Type II pneumococcus In other words the protective antibodies are type specific

The capsular polysaccharides of several types of pneumococcus have been prepared in relatively pure form as previously noted It has been shown that the protective capacity of a serum depends upon the amount of antibody present which is capable of reacting with the capsular polysaccharides By the addition of an appropriate amount of purified specific capsular polysaccharide to a highly protective serum all of the anticapsular antibodies can be precipitated and *removed A serum ab*sorbed in this way loses its protective power Anticapsular antibodies can also be absorbed from a serum by permitting it to react with whole encapsulated pneumococcal cells and here again the protective power is removed at the same time However one cannot conclude from the results of *absorption* with whole bac*terial cells that it is the* anticapsular antibodies that are of importance in protecting against infection since other antibodies reacting with other components of the pneumococcal cell, may be removed simultaneously On the other hand, absorption of serum with purified capsular polysaccharides shows unequivocally that it is the antibody to this material that is of importance in protection against infection and at the same time dem*onstrates the importance of the capsule in the* pathogenicity of the organism

Even more direct evidence for the importance of the capsule in pneumococcal pathogenicity is found in the fact that on immunizing with highly purified capsular polysaccharides man and mouse produce anti*bodies which give a precipitin reaction with* the homologous polysaccharides agglutinate encapsulated pneumococci and protect specifically against infection by the homologous type On the other hand immunization with R organisms results in production of serum of only slight protective power (Tillett, 1928) Anti *R sera do not agglutinate S organisms*

The loss of the capsule in R organisms deprives the cells of most of their virulence though not all With fully virulent encapsulated strains a dilution of broth culture containing from 1 to 5 viable organisms (1×10^{-8} ml of culture) will kill mice following intraperitoneal injection With R organisms selected from the same strain by growing it in anti S serum 0 5 to 1 0 ml of culture is necessary to cause death or approximately 100 000 000 to 200 000 000 living R organisms Therefore removal of the capsule has decreased virulence roughly 100 000 000 *(10^8) fold* In mice in the case of Types II III and VII virulence is related to the *quantity* of SSS formed by different strains of the same type (MacLeod and Krauss 1950) For example strains of Type II that produce small amounts of SSS II are less virulent for mice than strains that form larger amounts The quantity of *SSS synthesized by a strain is* under genetic control as shown by studies of mutants of different synthetic capacities and of the transformation reaction carried out with DNA extracts prepared from them

The antiphagocytic property of the pneumococcal capsule can be demonstrated in vitro

Sputum which is usually produced through out the disease is characteristically bloody or rusty the red cells being intimately mixed with the other components Large numbers of pneumococci are usually present and can be quickly identified by immunologic methods especially the Neufeld quellung or capsular swelling reaction

The onset of acute lobar pneumonia is characteristically sudden often with the occurrence of a chill and sharp pleural pain Recovery of untreated cases frequently occurs likewise with dramatic suddenness—the so called pneumonic crisis which generally occurs within 5 to 10 days from the initial chill

Pneumococci can be recovered from the blood in a variable proportion of cases the reported incidence of bacteremia depending in good part on the frequency with which cultures are made and the technic followed In the large series of cases compiled by Heffron (1939) bacteremia occurred in 26 per cent Persistent and increasing bacteremia are grave prognostic signs

Before the introduction of chemotherapy empyema was the commonest complication of lobar pneumonia occurring in approximately 3 per cent of patients (Heffron) Adequate antibiotic therapy has made it a rarity

The evidence indicates very strongly that pontaneous recovery from pneumococcal pneumonia is dependent upon the development of type speci ic antibody which can be demonstrated usually in the blood at about the time of crisis It should be remembered however that the antibody demonstrable in the circulating blood represents the excess left over after combination with type specific polysaccharide present in the lung and other organs of the body In other words antibody is being formed before recovery is apparent but appears in sufficient excess at that time so that it can be detected in the blood serum

LABORATORY DIAGNOSIS

The effective use of specific serum therapy for treatment of pneumococcal pneumonia necessitated rapid and accurate methods of etiologic diagnosis Of the many methods described the Neufeld quellung or capsular swelling reaction is the simplest and best Neufeld observed in 1902 that when pneumococci of a particular type and homologous antiserum are mixed together the capsules of the organisms become greatly swollen and in this state are clearly visible under the microscope The swelling is due to combination of pecific antibody with the capsular polysaccharide

Sputum is emulsified by drawing it repeatedly into a syringe Then it is stained by Gram s method to determine whether organisms having the morphology of pneumococci are present Loopfuls of emulsified sputum are mixed with a loopful of the various specific antipneumococcal sera a loopful of methylene blue is added to make the somatic portion of the cells more easily visible and the preparation is examined immediately under the oil immersion lens In a positive reaction the pneumococcal capsule has a greatly swollen appearance and its border is sharply delimited from the surrounding medium The Neufeld reaction can be used to identify pneumococci in putum in the exudates from experimentally infected animals and when grown in artificial culture media Under the latter circumstances young cultures should be examined since in older cultures much of the capsular material diffuses away from the pneumococci into the surrounding medium so that capsular swelling is less striking

Diagnosis can also be made by injecting mice intraperitoneally with sputum In general pneumococci are much more pathogenic for the mouse than other organisms present in sputum or saliva and consequently come to predominate within a few hours in both the peritoneal exudate and blood of the mouse Upon death of the mouse which usually occurs within 16 to 48 hours following injection of sputum some of the peritoneal exudate is removed examined microscopically after Gram staining and then typed by the Neufeld reaction Pneumococci can usually be recovered in pure culture from the heart blood of the infected mouse It is good practice to make cultures of the heart blood for confirmatory studies

Pneumococci can be typed also by agglutination and precipitin reactions but the simplicity and the great accuracy of the Neufeld reaction make it a preferable technic

Culture of the blood of patients with pneumonia is important from the diagnostic point of view but even more so as a *guide in* prognosis

from avirulent Type XIV pneumococcus become highly mouse virulent when transformed to pneumococcus Type II similarly when virulent Type II pneumococci are transformed to Type XIV by way of the R variant, the newly constituted Type XIV strain is as avirulent for the mouse as naturally occurring Type XIV pneumococci (MacLeod and McCarty 1942)

PATHOGENESIS OF PNEUMOCOCCAL INFECTION

Pneumococcal pneumonia is rarely a primary infection but usually follows damage to the respiratory tract caused by some unrelated agent whether viral or chemical In most instances pneumonia is preceded by an upper respiratory infection such as common cold or influenza Therefore pneumococcal pneumonia may be classed as a complex infection This relationship to viral infections probably explains why pneumococcal pneumonia is commonest during the cold months of the year in northern latitudes since common colds and influenza occur with greatest frequency at these times It is unlikely that the virulence of pneumococci for man varies with the seasons

It is apparent from the relation of pneumococcal pneumonia to viral infection that the normal respiratory mucosa must possess great natural resistance to pneumococcus especially when it is recalled that between 40 and 70 per cent of all normal humans are carriers of pneumococci many types of which are potentially virulent The factors normally involved in protection of the respiratory mucous membrane have not been defined and we are also ignorant of the nature of the predisposing injury caused by viruses or chemical irritants such as gases though it seems likely that actual destruction of the superficial cell layers may be important in permitting invasion of underlying tissues Recent evidence indicates that the possession of type specific antibody not only protects man against infection under natural conditions but also renders him less likely to become a carrier of pneumococci of homologous type (MacLeod et al 1945) However it is reasonably certain that in the main normal resistance to pneumococcal pneumonia does not depend upon the possession of antibodies reactive with the many types with which one comes in contact but rather upon other factors including the accident of whether one happens to be a carrier of a highly pathogenic type such as Type I or II

In the healthy adult, pneumococcal pneumonia characteristically involves one or more lobes or a discrete portion of them, leaving the remaining bronchopulmonary system relatively uninvolved In infants, in young children and in the aged, the lesions more commonly follow a bronchial distribution without the localized character of lobar pneumonia

Experimental pneumococcal pneumonia has been studied in the monkey by Blake and Cecil (1920) and in the dog by Robertson and others (see Terrell et al , 1933) In both species pneumonia can be produced successfully by administering a narcotic such as morphine to depress respiration and the cough reflex and then introducing virulent pneumococci into the trachea or directly into a portion of a pulmonary lobe by means of a fine catheter In the dog an important factor is the production of atelectasis in the portion of the lobe infected, and it seems likely that atelectasis may be important also in the etiology of lobar pneumonia in man Increased bronchial secretions and edema occurring during viral infections of the upper respiratory tract may play a significant part through plugging of bronchioles and the production of areas of atelectasis In addition the tendency for secretions to pool in the most dependent portions may explain in part why the lower pulmonary lobes are most frequently involved

PATHOLOGIC AND CLINICAL PICTURE

The lesion in the lung consists essentially of marked edema of the alveolar walls with an outpouring into the alveoli of fibrinous exudate containing large numbers of red blood cells and polymorphonuclear leukocytes Therefore the affected lung becomes consolidated The overlying pleura is involved early with a serous pleural effusion as a common incident Pneumococci in large numbers can be seen throughout the inflamed area, but despite their presence both in the alveoli and the alveolar septa necrosis does not take place The absence of necrosis of the alveolar septa explains why upon recovery from the disease the lung is able to return to its original state

Spontaneous recovery from pneumococcal pneumonia is associated with the appearance of specific antibody in the blood over and above the amount required to combine with the capsules of the organisms and thus render them susceptible to phagocytosis Therefore in the natural di ease there is a competition between the capacity of the pneumococci to grow and produce SSS and the ability of the infected p rson to form antibody to it Treat ment with specific antiserum tips the balance in favor of the host since sufficient can be given in a short time to combine with all the SSS present both that which is on the surface of the micro organisms and that which is free in the blood and the tissues

The sulfonamide drugs which are essen tially bacteriostatic act by restraining the growth of the organisms until sufficient anti body has been formed in the body to assure opsonization and phagocytosis of the pneumo-cocci The participation of specific immunity appears to be necessary for a successful out come in sulfonamide therapy and as might be expected in the experimental animal the sulfonamides and specific antisera exert a syn ergistic effect (MacLeod 1939)

In the case of penicillin which is a bac tericidal compound when used in full thera peutic dosage the development of specific antibody seems to play a much less significant part in recovery in the natural disease in man or in experimental infections of the highly susceptible mouse (MacLeod and Stone 1945) The role of a specific antibody re sponse in recovery following treatment with the highly bacteriostatic tetracycline com pounds has not been studied adequately

EPIDEMIOLOGY OF PNEUMOCOCCAL PNEUMONIA

As noted above pneumococcal pneumonia occurs almost always secondary to injury to the re piratory mucosa caused by an unrelated agent such as a virus infection or irritating gas Furthermore the virulence of the various pneumococcal types for man differs greatly Types I and II have the most pronounced human virulence since between them they cause about one half of all the cases of lobar pneumonia in the adult On the other hand certain other types are encountered very rarely as the cause of pneumonia and hence can be considered as of low human virulence It should be apparent therefore that the chances of developing pneumococcal pneumonia de pend in great part upon whether or not the nonimmune individual is a carrier of one of the more highly pathogenic pneumococcal types at the time he comes down with a viral infection of the respiratory tract such as com mon cold or influenza There is ample evi dence (summarized by Heffron 1939) that when pneumococcal pneumonia is epidemic in a community it is always associated with a high carrier incidence of the pneumococcal types causing disease Significantly most of the epidemics of pneumococcal pneumonia re ported in the literature have been caused by the same types that are responsible for most of the cases of endemic pneumonia Types I II IV V and VII Under normal circum stances the incidence of carriers of the highly pathogenic types is relatively low However if a high carrier incidence of pathogenic types prevails at a time when viral infections of the respiratory tract are epidemic epidemic pneu mococcal pneumonia is liable to occur also (Hodges and MacLeod 1946)

Chance would appear to determine whether the nonimmune individual becomes a carrier of one or more types of pneumococci (Hodges et al 1946) although the immune person is less capable of becoming a carrier than the nonimmune (MacLeod et al 1945)

Most of the reported epidemics of pneumo coccal pneumonia have occurred in relatively closed communities such as mental hospitals prisons and military installations The living conditions in such circumstances appear to favor the dissemination of the more patho genic types once the latter are introduced into the community In addition the incidence of pneumococcal pneumonia is higher in workers in certain occupations such as in steel mills and in coal mines than in the general popula tion (Heffron 1939)

There is evidence that the normal carrier is of more importance in the dissemination of the infective types than the patient ill with pneumonia (MacLeod et al 1945)

PREVENTION OF PNEUMOCOCCAL PNEUMONIA

From the observations cited in relation to the epidemiology of pneumococcal pneumonia

SPECIFIC SERUM THERAPY

Pneumococcal pneumonia is one of the few infectious diseases for which effective specific serum therapy has been evolved The intro duction of sulfonamide drugs in 1937 and the many antibiotics since that time have entirely displaced antiserum in therapy because they are more effective and much easier to use However, a description of serum therapy is given because of its historical interest as well as *the general principles which it illustrates* The basis for serum therapy was laid in the fundamental studies of Avery Chickering Cole and Dochez (1917) who first showed convincingly that the antiserum to be used must be type specific, that antibody must be given in adequate amounts and that it is most *effective when administered early in the course* of the disease Applied originally to treatment of pneumonia caused by Types I and II highly potent antisera later became available commercially for pneumonia caused by the majority of pneumococcal types (for sum maries see Bullowa 1937 and Lord and *Heffron* 1938)

The earliest antisera were prepared by the immunization of horses with killed pneumo cocci and were administered intravenously in unconcentrated form Subsequently various methods were developed for refining and con centrating the antibody globulins in the crude serum which greatly facilitated treatment The introduction of rabbits for preparing anti pneumococcal serum for therapeutic purposes was another advance since in general higher titers of antibody could be obtained (Horsfall et al 1937)

The dosage of type specific antipneumococ cal serum can be controlled by determining whether the patient s blood and tissues con tain an excess of antibody Specimens of blood serum obtained at intervals after treatment can be tested for free antibody by means of agglutination reactions with suspensions of homologous pneumococci Dosage of antiserum is adjusted so that an excess of antibody is constantly present *in the blood* However a more useful method is the skin test with the purified specific capsular polysaccharides de scribed by Francis (1933) 0 1 mg of ho mologous specific capsular polysaccharide dissolved in 0 1 ml of saline is injected intracutaneously If circulating antibody is present, a wheal and erythema reaction ap pears at the site of polysaccharide injection within 15 or 30 minutes The wheal and erythema are due to the combination locally of polysaccharide and homologous antibody As early as possible in the course of the dis ease antibody is administered intravenously in an amount sufficient to result in a positive polysaccharide skin test In most instances defervescence and the onset of recovery occur within a few hours after sufficient antibody has been given At intervals of a few hours after serum therapy was first given, the poly saccharide skin test is repeated in order to make sure that antibody remains present in excess

The ability to make a rapid etiologic diag nosis by means of the Neufeld reaction the preparation of highly concentrated prepara tions of specific antibody and control of dosage by the polysaccharide skin test together made the serum treatment of pneumococcal pneu monia highly effective On the other hand, the specialized nature of the technics involved the necessity for maintaining stocks of many type specific antisera and the constant fear of anaphylactic reactions following intravenous administration of foreign protein prevented the serum treatment of pneumococcal pneu monia from achieving general use

CHEMOTHERAPY

Of bacteria causing human infections pneu mococci are among the most susceptible to the action of sulfonamide compounds and various antibiotics Moreover with the exception of the sulfonamides the appearance of drug re sistance has been a rare occurrence during the course of therapy and up to the present time has caused little difficulty Resistance of pneu mococci to sulfonamides has been encountered especially in patients treated with inadequate dosage and in cases in which a purulent complication such as empyema was present Penicillin the tetracycline compounds and chloramphenicol are more effective agents in *treating pneumococcal infections and have* largely displaced the sulfonamides as the agents of choice in therapy

It is instructive to compare the mechanism of action of specific serum therapy and chemo therapy in combating pneumococcal infections

Spontaneous recovery from pneumococcal pneumonia is associated with the appearance of specific antibody in the blood over and above the amount required to combine with the capsules of the organisms and thus render them susceptible to phagocytosis Therefore in the natural disease there is a competition between the capacity of the pneumococci to grow and produce SSS and the ability of the infected p rson to form antibody to it Treatment with specific antiserum tips the balance in favor of the host since sufficient can be given in a short time to combine with all the SSS present both that which is on the surface of the micro organisms and that which is free in the blood and the tissues

The sulfonamide drugs which are essentially bacteriostatic act by restraining the growth of the organisms until sufficient antibody has been formed in the body to assure opsonization and phagocytosis of the pneumococci The participation of specific immunity appears to be necessary for a successful outcome in sulfonamide therapy and as might *be expected in the experimental animal the* sulfonamides and specific antisera exert a synergistic effect (MacLeod 1939)

In the case of penicillin which is a bactericidal compound when used in full therapeutic dosage the development of specific antibody seems to play a much less significant part in recovery in the natural disease in man or in experimental infections of the highly susceptible mouse (MacLeod and Stone 1945) The role of a specific antibody response in recovery following treatment with the highly bacteriostatic tetracycline compounds has not been studied adequately

EPIDEMIOLOGY OF PNEUMOCOCCAL PNEUMONIA

As noted above pneumococcal pneumonia occurs almost always secondary to injury to the respiratory mucosa caused by an unrelated agent such as a virus infection or irritating gas Furthermore the virulence of the various pneumococcal types for man differs greatly Types I and II have the most pronounced human virulence since between them they cause about one half of all the cases of lobar pneumonia in the adult On the other hand certain other types are encountered very rarely as the cause of pneumonia and hence can be considered as of low human virulence It should be apparent therefore that the chances of developing pneumococcal pneumonia depend in great part upon whether or not the nonimmune individual is a carrier of one of the more highly pathogenic pneumococcal types at the time he comes down with a viral infection of the respiratory tract such as common cold or influenza There is ample evidence (summarized by Heffron 1939) that when pneumococcal pneumonia is epidemic in a community it is always associated with a high carrier incidence of the pneumococcal types causing disease Significantly most of the epidemics of pneumococcal pneumonia reported in the literature have been caused by the same types that are responsible for most of the cases of endemic pneumonia Types I II IV V and VII Under normal circumstances the incidence of carriers of the highly pathogenic types is relatively low However if a high carrier incidence of pathogenic type prevails at a time when viral infections of the respiratory tract are epidemic epidemic pneu*mococcal pneumonia is liable to occur also* (Hodges and MacLeod 1946)

Chance would appear to determine whether the nonimmune individual becomes a carrier of one or more types of pneumococci (Hodges et al 1946) although the immune person is less capable of becoming a carrier than the nonimmune (MacLeod et al 1945)

Most of the reported epidemics of pneumococcal pneumonia have occurred in relatively closed communities such as mental hospitals prisons and military installations The living conditions in such circumstances appear to favor the dissemination of the more pathogenic types once the latter are introduced into the community In addition the incidence of pneumococcal pneumonia is higher in workers in certain occupations such as in steel mills and in coal mines than in the general population (Heffron 1939)

There is evidence that the normal carrier is of more importance in the dissemination of the infective types than the patient ill with pneumonia (MacLeod et al 1945)

PREVENTION OF PNEUMOCOCCAL PNEUMONIA

From the observations cited in relation to the epidemiology of pneumococcal pneumonia

it seems likely that control can be achieved either by preventing the nonbacterial respiratory infections which predispose or else by specific prophylaxis of pneumococcal infections themselves A certain amount of success has been achieved through both approaches

In recent years evidence has been presented that the incidence of influenza can be greatly reduced by immunization with vaccines of influenza virus types A and B, provided that the strains of virus present in the vaccine are closely related immunologically to the strains of virus causing epidemic influenza Therefore improvement in influenza vaccines and their general use might be expected to cause a reduction not only in influenza but also in pneumococcal pneumonia which occurs secondary to influenza Similarly the development of specific immunizing preparations for other viral infections of the respiratory tract should lead to a concomitant reduction in pneumococcal infections

Repeated attempts have been made to immunize against pneumococcal pneumonia (for a critical review see Heffron, 1939) In the earlier trials the vaccines consisted of heat killed pneumococci which were injected subcutaneously Although proof was lacking the general opinion of those who employed whole bacterial vaccines was that a beneficial result was obtained The evidence of Lister and Ordman (1935) in South Africa was especially suggestive of a prophylactic effect In more recent years preparations of the capsular polysaccharides have been used following the demonstration by Francis and Tillett in 1930 that the isolated polysaccharides are antigenic for man Again suggestive evidence was obtained especially through the studies of Felton (Ekwurzel et al 1938) that immunization of man with the capsular polysaccharides prevents pneumococcal pneumonia Most of the studies on antipneumococcal immunization have been inadequate in one or more respects especially because of failure to determine whether the apparent reduction in pneumonia was confined to the types against which immunization was practiced as well as failure to design the experiment so that adequate controls were included

Most of the deficiencies inherent in previous attempts at immunization against pneumococcal pneumonia appear to have been avoided in the studies reported by MacLeod Hod es Heidelberger and Bernhard (1945), who have presented what may be considered as reasonably conclusive evidence that immunization by subcutaneous injection of purified capsular polysaccharides can prevent pneumococcal pneumonia

Immunization was carried out in an Army camp where Types I, II, IV, V, VII and XII pneumococcal pneumonia had been epidemic for 2 years A dose of 0 06 mg of each of the capsular polysaccharides of pneumococcus Types I II, V and XII was injected subcutaneously into half the population the remainder serving as controls In the immunized men occurrence of pneumonia caused by Types I, II, V and XII ceased within 2 weeks after immunization but continued in the non immunized controls The incidence of pneumonia caused by pneumococcal types other than those represented in the vaccine was not affected in either group

Although Types I II V and VII pneumococci continued to cause disease in the non immune group, the incidence was not so high as was expected on the basis of the previous 2 years experience moreover the incidence of pneumonia caused by Type XII and other types was unaffected Therefore it is likely that immunization of one half the population protects not only those who are immunized but also affords a measure of protection to the nonimmune segment Partial protection of the nonimmune portion of the population may be explained by the observation that the immunized individual is less capable of carrying homologous pneumococci in the pharynx than the nonimmune and because of the reduction in carriers dissemination of pneumococci is reduced

Large scale immunization of a civilian population by means of specific polysaccharides has not been employed for the prevention of pneumococcal pneumonia

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10

The Hemolytic Streptococci

The streptococci comprise a large and biologically diverse group of micro organisms The principal criteria for classifying them are morphologic In general streptococci can be *defined as gram positive organisms* spherical or oval in shape that tend to grow in chains With the exception of the pneumococci which are usually considered separately by medical bacteriologists because of their unique biologic characteristics those streptococci which possess primary pathogenicity for man and animals belong to the subdivision of hemolytic streptococci The nonhemolytic streptococci are saprophytic and many species can be found among the flora of the upper respiratory and the intestinal tracts of normal individuals They are sometimes found in association with infectious processes most commonly when some predisposing structural abnormality is present as in bacterial endocarditis urinary tract infections or secondary wound infec*tions*

Hemolytic streptococci cause the lysis of mammalian red blood cells a property which is useful in the initial isolation and identification of these organisms in culture material As a result of extensive serologic studies they have been divided into a number of distinct serologic groups which are related in some degree to the natural habitat and the pathogenicity of the organism Accordingly serologic group has assumed primary importance in the identification of pathogenic streptococci It has become apparent that hemolytic streptococci of a single group group A, are *not only* responsible for most of the acute streptococcal disease of man but also are involved in the initiation of late sequelae such as rheumatic fever and glomerulonephritis

HISTORY

Streptococci were first seen in materials obtained from certain human infections Thus Billroth (1874) described globular chains of micro organisms in erysipelas and wound infections and Pasteur (1879) demonstrated similar organisms in the blood of a patient with puerperal sepsis Ogston (1881) isolated cocci growing in chains from acute abscesses and used animal experiments to demonstrate the pathogenicity of the organisms after they had been grown on artificial media Following the demonstration by Koch (1881) that streptococci were present with great regularity in excised erysipelas lesions Fehleisen (1882 1883) grew the organism in pure culture from such areas and induced typical erysipelas in human beings with subcultures

Organisms with similar characteristics were subsequently obtained from a wide variety of materials and considerable difficulty arose concerning their identification and classification Inevitably many streptococci were considered to be specific for the disease entity in which they appeared and as a result such names as *Streptococcus erysipelatis* and *Streptococcus scarlatinae* came into use Attempts to arrive at a system of classification by studying biochemical and physiologic properties *such as the pattern of fermentation* of various carbohydrates did not prove to be of great value, since no clear relationship between these

findings and disease producing capacity could be established The differentiation of strepto cocci into hemolytic green and indifferent strains based upon their action on red blood cells in vitro was first proposed by Schott muller (1903) In his elaboration of this tech nic Brown (1919) introduced the terms alpha beta and gamma to describe slight hemolysis with greening frank hemolysis or no effect on erythrocytes respectively It was recognized early that pathogenic streptococci for the most part fell in the truly hemolytic (beta) category

A useful system for the classification of hemolytic streptococci was gradually evolved by the application of serologic technics Al though not historically the first step a devel opment of primary importance in bringing order into the study of streptococci came with the separation of these organisms into well defined serologic groups by Lancefield (1933) It was clear that most strains associated with human infections belonged to a single sero- logic group designated group A The occur rence of serologically differentiable types among human strains had been demonstrated earlier by agglutination and mouse protection tests (Dochez Avery and Lancefield 1919) The further development of type differentia tion of human strains was pursued by different approaches in two laboratories Lancefield (1928a) devised a precipitin technic for typing based on the use of extracts containing type specific protein antigens and Griffith (1927 1934) classified streptococcal strains by ap plying a slide agglutination technic Informa tion obtained from the grouping and the typ ing of hemolytic streptococci established the fact that the use of disease specific names is not justified since group A strains of a single type can be recovered from a variety of dis ease entities More important the serologic classification of streptococci supplied the tools required for a detailed study of the bacteri ology the immunology and the epidemiology of streptococcal disease

MORPHOLOGY

The outstanding morphologic characteristic of streptococci is their tendency to grow in chains At certain stages of the growth cycle the individual cocci are spheroid However during growth of the organisms prior to divi sion they become elongated on an axis parallel with that of the chain and assume an ovoid appearance The average diameter of the cocci is approximately 1 micron although there is considerable variation in size and certain minute streptococci may be one fourth to one half this size When growing under unfavorable conditions bizarre forms frequently appear with irregularities in the size the shape and the gram positivity of cocci even within a single chain In young actively growing cultures in favorable media such as serum or blood broth the cocci are uniform and the chains appear in their most character istic form

After elongation during growth the cocci divide in a plane perpendicular to the long axis of the chain Because the individual cocci divide into pairs a diplococcoid appearance of the members of a chain is often quite striking Chaining results from the fact that a connect ing link between the cocci probably composed of material like that forming the cell wall is retained following division These intercellular bridges are not easily broken and withstand such procedures as shaking however the chains can be disrupted without extensive de struction of cocci by subjecting them to brief periods of sonic oscillation (Slade and Slamp 1956) The length of the chains varies within wide limits and is influenced to some degree by the nature of the culture medium Many hemolytic streptococci grow in relatively short chains of 8 to 10 members others much longer Occasionally one will find extremely long chains suggesting that each individual chain of the inoculum has continued to in crease in length without disrupting during growth This type of chain formation is more common among certain of the nonhemolytic streptococci

The chain forming property has an impor tant bearing on the interpretation of quantita tive studies on the growth of streptococci The usual technic for determining the number of viable bacterial cells depends upon counting the colonies formed when dilutions of the cul ture are incorporated in an appropriate agar medium In the case of streptococci this pro cedure provides an estimate not of the number of individual viable cells but of the number of intact chains

Capsule formation is characteristic of many

group A streptococci, and capsules of similar composition are formed by certain members of group C These capsules are composed largely of hyaluronic acid, which is either identical with or very closely related to the hyaluronic acid found in mammalian tissues Capsules are most readily demonstrated microscopically in very young actively growing cultures in enriched media With continued growth the hyaluronic acid is released into solution in the medium and capsules are no longer demonstrable The capsules are seen most easily in wet preparations prepared by the India ink technic and appear as a large clear zone surrounding the chains, frequently several times the diameter of the cocci

Capsulation in group B streptococci is quite different in nature and depends on the occurrence of type specific polysaccharide comparable with those elaborated by pneumococci

COLONY FORMS

The colonial characteristics of hemolytic streptococci are usually studied on the surface of blood agar plates so that the pattern of hemolysis and colony morphology can be observed simultaneously The size the distinctness and the rate of development of the zone of hemolysis surrounding surface colonies varies considerably Some strains notably certain members of groups C and D produce unusually large and brilliant zones while others may show no more than a narrow ring of hemolysis surrounding the colony The species of blood used in the agar medium influences the nature of the hemolysis and streptococci *occur which give clear hemolysis with one* mammalian blood but cause no hemolysis or only greening with others

Not all streptococci which fall into the recognized serologic groups produce hemolysis on the surface of blood agar plates Thus certain members of groups B C D H K and O and all members of group N are essentially nonhemolytic Even among group A strains variants have been encountered which lack the ability to produce streptolysin S and thus are nonhemolytic under the aerobic conditions of surface growth Such strains produce the oxygen labile streptolysin O so that they become hemolytic when grown anaerobically or as deep colonies in agar pour plates

Isolated colonies of streptococci on blood agar average 1 to 2 mm in diameter The so called minute streptococci, including members of groups F and G, form much smaller colonies The topography of the colonies does not follow a single well defined pattern, they are usually circular in outline with smooth or slightly serrated edges and a rounded surface

Among group A streptococci 3 distinct colony forms are recognized mucoid matt and glossy The mucoid character is associated with the production of hyaluronic acid capsules and the most strikingly mucoid strains are those which form abundant amounts of the polysaccharide with large well developed capsules Mucoid colonies are best observed on fresh blood agar plates or plates that have been sealed to prevent loss of moisture They tend to be large and glistening and may have the appearance of droplets of water although when touched with a loop the viscous character of the colony is readily apparent The suggestion of fluidity is increased by the fact that the colonies frequently do not retain a circular shape They often collapse as a result of drying, leaving a flattened colony with an irregular surface

Matt colonies are relatively large and flat with a granular or stippled surface and may present an appearance similar to that of the collapsed mucoid colony Glossy colonies on the other hand are smaller usually more rounded and have a shiny reflecting surface

There is a limited relationship between colony form and production of the antigenic constituents responsible for type specificity and virulence Strains which produce mucoid or matt colonies usually elaborate M protein and are potentially virulent, while those which produce glossy colonies contain little or no M protein and are of low virulence However there are many exceptions to this relationship and colony form cannot be used as a reliable indication of either M protein production or potential virulence Thus nonvirulent strains can produce hyaluronic acid and give mucoid colonies and M protein may be produced by organisms which form typically glossy colonies

GROWTH REQUIREMENTS

The pathogenic hemolytic streptococci are among the most fastidious of micro organisms in growth requirements As a routine proce

dure they are usually cultivated in complex media composed of peptone meat infusion salts and glucose Heating may reduce the ability of a medium to support growth and the best results are obtained when the broth is sterilized by filtration With even the most satisfactory media of this type further enhancement of growth usually can be achieved by the addition of blood or serum The effect of blood or serum is especially striking in the case of solid media since the growth of streptococci on the surface of agar plates tends to be quite poor in the absence of these additives

Some information concerning the minimal requirements for the growth of group A streptococci has been obtained by the use of chemically defined media (Bernheimer et al 1942 Slade et al 1951) although very few strains will grow vigorously and reproducibly under these conditions The findings indicate that up to 15 amino acids and almost all of the known members of the vitamin B complex are needed In addition purines and pyrimidines are required for certain strains and asparagine and glutamine may increase the amount of growth significantly The reducing conditions necessary for the initiation of growth in these media are usually obtained by the addition of a sulfhydryl compound such as cysteine or thioglycollate

There is a certain amount of evidence to suggest that the missing substances which account for the inadequacy of defined media are peptide in nature The substance designated streptogenin (Woolley 1941) which was found to be necessary for the growth of certain group A streptococcal strains is apparently a family of peptides rather than a single substance Others have found that peptide preparations are stimulatory to the growth of group A streptococci in defined media (Slade 1954)

Theoretically investigation of the extracellular enzymes and toxins of group A streptococci would be carried out most satisfactorily with defined media because of the absence of macromolecular constituents not arising from the organisms However these media have proved to be generally unsatisfactory for this purpose and the alternative approach of employing only the dialyzable components of complex meat infusion peptone media has been adopted (Stock 1939 Dole 1946) Most strains grow well and produce the expected complement of extracellular substances in appropriate dialysate media

The hemolytic streptococci are facultative anaerobes and metabolize glucose with the formation of lactic acid The accumulation of lactic acid with the attendant fall in pH is a limiting factor in the growth of the organisms in an otherwise adequate medium as indicated by the fact that massive growth can be obtained in the presence of excess glucose if the lactic acid formed is continuously neutralized Growth is optimal at 37° C and is markedly inhibited at temperatures above 40° C

GROUP DIFFERENTIATION

The division of hemolytic streptococci into serologic groups depends upon the occurrence of group specific cellular antigens which are carbohydrate in nature and are designated as C carbohydrates (Lancefield 1928b 1933) The presence of these antigens is demonstrated by means of precipitin reactions between solutions of the carbohydrate extracted from streptococcal cells and antisera prepared by immunizing rabbits with heat killed suspensions of streptococci Lancefield showed that separating hemolytic streptococci into distinct categories by this technic resulted in a classification related to the most characteristic source of the organisms Thus group A is composed of strains usually pathogenic for man group B of strains from bovine mastitis group C is commonly found in streptococcal diseases of lower animals etc The source and the characteristics of the common serologic groups—including those that have been added since the initial studies of Lancefield—are summarized in Table 30 The relationship between group specificity and habitat is not a rigid one Thus although group A strains are responsible for most human streptococcal infections occasionally representatives of all other groups may be isolated from human sources

The serologic groups listed in Table 30 embrace the great majority of strains of hemolytic streptococci However additional strains are encountered which possess the general properties of hemolytic streptococci but do not fall into any of the defined groups

Information concerning the cellular localization and the chemical composition of the group specific carbohydrates has been obtained

TABLE 30 SUMMARY OF RECOGNIZED SEROLOGIC GROUPS OF STREPTOCOCCI

GROUP	USUAL HABITAT	USUAL PATHOGENICITY
A	Man	Many human diseases
B	Cattle	Mastitis
C	Many animals	Many animal diseases
	Man (human strains)	Mild respiratory infections
D	Dairy products	Genito urinary tract infections endocarditis wound infections
	Intestinal contents of man and animals (enterococci)	
E	Normal milk	None known
	Swine	Pharyngeal abscesses of swine
F	Man	Questionable found in respiratory tract
G	Man	Mild respiratory infections rare
	Dogs	Genital tract infections in dogs
H	Man	Questionable found in respiratory tract
K	Man	Questionable found in respiratory tract
L	Dogs	Genital tract infections
M	Dogs	Genital tract infections
N	Dairy products	None
O	Man	Occur in upper respiratory tract but not associated with disease Endocarditis

Groups A to E were described by Lancefield (1933) groups F and G by Lancefield and Hare (1935) groups H and K by Hare (1935) and groups L and M by Fry (unpublished) Group N was identified independently by several groups and the letter N was assigned by Shattock and Mattick (1943) Group O was described by Boissard and Wormald (1950)

in recent years The carbohydrate is an integral component of the bacterial cell wall and may account for as much as 10 per cent of the dry weight of the organism This has been most clearly established in the case of group A (McCarty 1952 Salton 1953) but there is adequate evidence to indicate that the same localization holds for the other groups The monosaccharide composition of the cell wall carbohydrates of the several groups follows a remarkably uniform pattern which supports the concept of a basic interrelationship between the hemolytic streptococci In all groups that have been examined (groups A through G) rhamnose and glucosamine are the major carbohydrate constituents of the cell wall (Cummins and Harris 1956) All of them contain the substituted hexosamine muramic acid which appears to be present in the cell walls of most gram positive species In addition other monosaccharides are found in certain groups, providing an adequate chemical basis for serologic differences

The occurrence of the group specific carbohydrates as a part of the essentially insoluble cell wall structure explains the need for relatively drastic procedures in obtaining solutions of the carbohydrates It is necessary to bring about partial disintegration of the cell wall with the release of soluble carbohydrate in a form that retains its serologic reactivity In practice 4 different methods have been employed for this purpose (1) Extraction of cells at pH 2 and 100° C (Lancefield 1928 a b) This method has the distinct advantage of simultaneously providing M protein containing extracts suitable for typing of group A strains (2) Extractions of cells with formamide at 160° 180° C (Fuller 1938) (3) Lysis of streptococci with enzymes derived from *Streptomyces albus* (Maxted 1948) The cell wall is actually dissolved by this procedure consequently the highest yields of carbohydrate are obtained (4) Autoclaving of suspensions of cells at 15 pounds presure for 15 minutes (Rantz and Randall 1955) Characteristically solutions of the carbohydrate react rapidly with specific antisera to give floccules of antigen antibody precipitate and show little or no cross reaction with heterologous group antisera Thus with an appropriate supply of antisera the serologic group of unknown strains can be quickly determined

A nonserologic method of differentiating

group A from the other groups of streptococci has been suggested by Maxted (1953) Group A strains were shown to be significantly more sensitive to bacitracin than strains of all other groups and by choosing the appropriate con centration of the antibiotic Maxted devised a simple plate method for tentative identifica tion of group A strains In a survey of a large series of strains the accuracy of this technic proved to be very high although rare excep tions were encountered

Streptococcal bacteriophages have been known for many years but the earlier studies were carried out for the most part without reference to serologic grouping Recent studies have shown that several group A phages are related serologically to one another but dis tinct from group C phage furthermore that susceptibility of streptococci of these two groups is primarily a group specific phenom enon (Krause 1957) although other factors influence the susceptibility of group A strains (Maxted 1955) The cell wall carbohydrate of group C strains inactivates group C phage suggesting that the group specific carbohydrate may serve as a phage receptor site in this case but no similar effect can be demonstrated with group A carbohydrate Also of interest with respect to the cell wall structure of strepto cocci is that fact that phage lysates of group C streptococci contain a lytic factor presuma bly an enzyme which is highly active on group A and group E strains as well as on group C (Maxted 1957 Krause 1957) This enzyme dissolves the cell wall with release of the group specific carbohydrate in much the same way as the *Streptomyces albus* enzymes

ANTIGENIC AND BIOCHEMICAL COMPOSITION OF GROUP A STREPTOCOCCI

Because of the importance of group A strep tococci in human infections the composition of these organisms has been studied in great detail Of particular importance are the sev eral surface antigens and the wide variety of extracellular products which are released into the environment during growth Not all of these antigens are limited in their occurrence to group A streptococci and substances simi lar to or identical with certain of these com ponents are produced by some members of other groups

SURFACE ANTIGENS

Group Specific Carbohydrate As noted above the group A carbohydrate is the major component of the bacterial cell wall Purified preparations of the carbohydrate contain a small amount of amino acid or peptide nitro-gen regardless of the method of extraction from the cell wall but serologic activity is referable to the sugar moiety The monosac charide constituents are rhamnose and hexo-samine (Schmidt 1952 Salton 1953 Mc Carty 1952) Most of the hexosamine occurs as N acetyl glucosamine although some is also present in the form of the acid substituted glucosamine muramic acid The dominant ser ologic specificity of the carbohydrate appears to depend upon side chains of N acetyl gluco samine (McCarty 1956) These side chains can be removed selectively by an induced enzyme obtained from a soil bacillus with concomitant loss of reactivity with group A antisera

In the course of passage of group A strains through animal hosts variants have been isolated which appear to have lost their group-specific carbohydrates since extracts do not react with group A antisera (Wilson 1945 Lancefield and Perlmann 1952b) These vari ants contain a cell wall carbohydrate composed of the same monosaccharides as the group A carbohydrate although they are present in different proportions (McCarty and Lance field 1955) Both the chemical and the sero logic differences of the variant carbohydrate are explained by the total absence of side chains of N acetyl glucosamine which deter mine group A specificity The chemical basis for reactivity of variant carbohydrate with homologous antisera is a rhamnose linkage which is also present in group A carbohydrate but is ordinarily masked by the N acetyl glu cosamine side chains (McCarty 1956)

Despite the fact that the group-specific carbohydrate occurs near the surface of the cell in the cell wall it does not appear to par ticipate in any of the serologic reactions of intact cells such as agglutination Aggluttina tion reactions have been shown to be depend ent upon protein antigens occurring at the cell surface

M Protein The M proteins are the most important of the surface proteins They are the antigens which determine the type spec

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A nonserologic method of differentiating

group A from the other groups of streptococci has been suggested by Maxted (1953) Group A strains were shown to be significantly more sensitive to bacitracin than strains of all other groups and by choosing the appropriate concentration of the antibiotic Maxted devised a simple plate method for tentative identification of group A strains In a survey of a large series of strains the accuracy of this technic proved to be very high although rare exceptions were encountered

Streptococcal bacteriophages have been known for many years but the earlier studies were carried out for the most part without reference to serologic grouping Recent studies have shown that several group A phages are related serologically to one another but distinct from group C phage furthermore that susceptibility of streptococci of these two groups is primarily a group specific phenomenon (Krause 1957) although other factors influence the susceptibility of group A strains (Maxted 1955) The cell wall carbohydrate of group C strains inactivates group C phage suggesting that the group specific carbohydrate may serve as a phage receptor site in this case but no similar effect can be demonstrated with group A carbohydrate Also of interest with respect to the cell wall structure of streptococci is that fact that phage lysates of group C streptococci contain a lytic factor presumably an enzyme which is highly active on group A and group E strains as well as on group C (Maxted 1957 Krause 1957) This enzyme dissolves the cell wall with release of the group specific carbohydrate in much the same way as the *Streptomyces albus* enzymes

ANTIGENIC AND BIOCHEMICAL COMPOSITION OF GROUP A STREPTOCOCCI

Because of the importance of group A streptococci in human infections the composition of these organisms has been studied in great detail Of particular importance are the several surface antigens and the wide variety of extracellular products which are released into the environment during growth Not all of these antigens are limited in their occurrence to group A streptococci and substances similar to or identical with certain of these components are produced by some members of other groups

Surface Antigens

Group Specific Carbohydrate As noted above the group A carbohydrate is the major component of the bacterial cell wall Purified preparations of the carbohydrate contain a small amount of amino acid or peptide nitrogen regardless of the method of extraction from the cell wall but serologic activity is referable to the sugar moiety The monosaccharide constituents are rhamnose and hexosamine (Schmidt 1952 Salton 1953 McCarty 1952) Most of the hexosamine occurs as N acetyl glucosamine although some is also present in the form of the acid substituted glucosamine muramic acid The dominant serologic specificity of the carbohydrate appears to depend upon side chains of N acetyl glucosamine (McCarty 1956) These side chains can be removed selectively by an induced enzyme obtained from a soil bacillus with concomitant loss of reactivity with group A antisera

In the course of passage of group A strains through animal hosts variants have been isolated which appear to have lost their group specific carbohydrates since extracts do not react with group A antisera (Wilson 1945 Lancefield and Perlmann 1952b) These variants contain a cell wall carbohydrate composed of the same monosaccharides as the group A carbohydrate although they are present in different proportions (McCarty and Lancefield 1955) Both the chemical and the serologic differences of the variant carbohydrate are explained by the total absence of side chains of N acetyl glucosamine which determine group A specificity The chemical basis for reactivity of variant carbohydrate with homologous antisera is a rhamnose linkage which is also present in group A carbohydrate but is ordinarily masked by the N acetyl glucosamine side chains (McCarty 1956)

Despite the fact that the group-specific carbohydrate occurs near the surface of the cell in the cell wall it does not appear to participate in any of the serologic reactions of intact cells such as agglutination Agglutination reactions have been shown to be dependent upon protein antigens occurring at the cell surface

M Protein The M proteins are the most important of the surface proteins They are the antigens which determine the type spec

ificity of group A streptococci, and in addition they have been shown to be an important factor in virulence Their role in virulence is comparable with that of the capsular polysaccharides in the virulence of pneumococci That is they serve to inhibit phagocytosis of the organisms by host leukocytes and this antiphagocytic effect is nullified by specific antibody There are more than 40 recognized types of group A streptococci, each characterized by a serologically distinct M protein It is indicated both by animal experiments and by observation of natural human infections that protective immunity is directed against the M protein and is therefore type specific The M proteins are firmly attached to the cell surface After mechanical disintegration of streptococci they remain associated with the isolated cell walls Removal of the protein in a soluble and serologically active form from either the intact cell or the cell wall is accomplished by the relatively vigorous procedure of boiling at pH 2 The M proteins of the various types of group A streptococci have many properties in common such as their resistance to heat in acid solution and their great susceptibility to proteolytic digestion The susceptibility of M protein to proteolytic enzymes is a property of the native protein and not dependent on previous treatment with acid and heat since it has been shown that this antigen can be destroyed by trypsin treatment of viable streptococci without killing the cells (Lancefield 1943) This fact provides additional evidence for the localization of M protein at the surface of the cell

The typing of group A streptococci is accomplished best by means of a precipitin reaction between an M containing extract of the organism and type specific rabbit antiserum Of necessity this means that there must be available a collection of a large number of different antisera Unfortunately M protein is not a highly antigenic substance and prolonged immunization with suitable streptococcal vaccines is often necessary in order to produce adequate amounts of precipitating antibody The antisera obtained must be absorbed with organisms of heterologous types to remove antibodies to common streptococcal antigens which might give cross reactions Because of these considerations typing of streptococci is not widely used as a routine bacteriologic diagnostic procedure However, it has proved to be of great importance in special clinical and epidemiologic studies

The detection of antibody to M proteins in human sera poses a special problem The ordinary in vitro tests such as the precipitin reaction are not sufficiently specific for this purpose, since M antigen preparations contain other streptococcal substances which give rise to a high incidence of cross reactions As a result, the measurement of type specific antibody must depend on complex biologic tests based on the role of the M antigen in virulence and phagocytosis For example it is often possible to demonstrate type specific protection with human sera in mouse infections, but this procedure is somewhat insensitive and requires relatively large amounts of M antibody The most useful procedure is the so called bactericidal test which exploits the fact that usually streptococci are rapidly killed following phagocytosis Streptococci containing M protein resist phagocytosis by human leukocytes and grow vigorously in normal blood However, in the presence of type specific antibody phagocytosis is greatly enhanced and virulent streptococci inoculated into blood if not present in excessive numbers can be completely destroyed by leukocytes Quantitative procedures for carrying out phagocytic tests have been devised and have been applied not only to the measurement of M antibody (Kuttner and Lenert 1944, Rothbard 1945) but also to the antigenic analysis of streptococci (Maxted 1956 Lancefield 1957)

Other Surface Protein Antigens In addition to the M protein there are other protein antigens which occur at or near the cell surface and apparently are attached to the cell wall Those which have been studied in detail and are characterized to some degree serologically and chemically have been designated T antigen and R antigen (Lancefield 1940 Lancefield and Perlmann, 1952b) A comparison of the properties of M T and R antigens is presented in summary form in Table 31

T antigens are present in most strains of group A streptococci and as in the case of the M antigens they are represented by a large number of serologically distinct proteins (see summary in Lancefield 1954) However the distribution of T antigens is not directly re

TABLE 31 COMPARISON OF SURFACE PROTEIN ANTIGENS OF GROUP A HEMOLYTIC STREPTOCOCCI*

	M ANTIGENS	T ANTIGENS	R ANTIGEN
Extraction procedure	Heating at pH 2 3	Proteolytic digestion	Tryptic digestion or heating at pH 7 8
Effect of proteolytic enzymes	Rapidly digested	Resist digestion	Resists trypsin Susceptible to pepsin
Iso electric point	pH 5 3 (Type 1)	Approx pH 4 5 (Type 1)	pH 4 5
Heat (at acid pH)	Stable	Labile	Destroyed slowly
Occurrence	Variable cellular component Often lost on cultivation in artificial media and gained on mouse passage	Constant component of a given strain present in both matt and glossy variants	Present in only a few types (Also found in certain strains of groups B C and G)
Specificity	Distinct M antigen for each type	One T antigen may be common to several types *some apparently* restricted to single type	Only one R antigen known Occurs in at *least 3 M types*
Antigenicity in rabbits	Moderately to weakly antigenic in intact cell Weakly antigenic in solution	Good antigen both in intact cell and in solution	Good antigen both in intact cell and in solution
Relationship to virulence	One of the essential factors in virulence Antibodies confer type specific protection	None known Antibodies not protective	None known Antibodies not protective

* Lancefield and Dole 1946 Lancefield and Perlmann 1952 a and b

lated to that of the M antigens In some instances a common or closely related T antigen is found in several different specific M types For example types 15 17 19 23 30 and 47 appear to have closely related T antigens In addition within a single M type certain strains may have one T antigen while others have a totally unrelated T antigen and some strains may have no demonstrable T antigen

The T proteins are resistant to proteolytic digestion and serologically active preparations have been obtained in soluble form by extracting streptococci with proteolytic enzymes Because they are readily destroyed by heat at acid pH ordinarily these antigens are not represented in M containing extracts Streptococci are agglutinated by antisera containing homologous T antibodies and T antigens have been studied most extensively by this technic Determination of T antigen by agglutination technics can occasionally be a useful adjunct in the identification of streptococci particularly in those cases where little or no M antigen appears to be present However since the T antigens have no known relationship to virulence or protection identification of this component does not have the same significance as determination of the specific M type

The R antigen is a third surface protein of group A streptococci which can participate in agglutination reactions but in this case only a single such antigen is known (Lancefield and Perlmann 1952b) It resists tryptic digestion but is destroyed by pepsin Because it occurs in relatively large amounts in certain strains and is only slowly destroyed by heat and acid pH originally it was confused with the type specific antigen and strains containing it were designated type 28 It has now been demonstrated that R antigen like T antigen has no detectable relationship to virulence and that strains which produce R protein fall into 3 M types (types 2 28 and 48) (Lancefield 1957)

In view of the complexity of the antigenic structure of group A streptococci it seems probable that there are other as yet unidentified surface antigens However the extensive serologic studies suggest that the M T

ificity of group A streptococci and in addition they have been shown to be an important factor in virulence Their role in virulence is comparable with that of the capsular polysaccharides in the virulence of pneumococci That is they serve to inhibit phagocytosis of the organisms by host leukocytes and this antiphagocytic effect is nullified by specific antibody There are more than 40 recognized types of group A streptococci each characterized by a serologically distinct M protein It is indicated both by animal experiments and by observation of natural human infections that protective immunity is directed against the M protein and is therefore type specific The M proteins are firmly attached to the cell surface After mechanical disintegration of streptococci they remain associated with the isolated cell walls Removal of the protein in a soluble and serologically active form from either the intact cell or the cell wall is accomplished by the relatively vigorous procedure of boiling at pH 2 The M proteins of the various types of group A streptococci have many properties in common such as their resistance to heat in acid solution and their great susceptibility to proteolytic digestion The susceptibility of M protein to proteolytic enzymes is a property of the native protein and not dependent on previous treatment with acid and heat, since it has been shown that this antigen can be destroyed by trypsin treatment of viable streptococci without killing the cells (Lancefield 1943) This fact provides additional evidence for the localization of M protein at the surface of the cell

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TABLE 32 EXTRACELLULAR PRODUCTS OF GROUP A STREPTOCOCCI

	INHIBITION BY SPECIFIC ANTIBODY	DISTRIBUTION	COMMENT
Erythrogenic toxin	+	Produced by most strains	Evidence for at least 3 serologically different toxins
Streptolysin S	−	Produced by most strains	In vitro production enhanced by serum or by polyribonucleotides
Streptolysin O	+	Produced by most strains	Active in reduced state only
Diphosphopyridine nucleotidase	+	Production more common among some serologic types than others	
Streptokinase	+	Produced by most strains	
Desoxyribonuclease A	+	One or more produced by all strains	Three erologically distinct enzymes which attack the same substrate
Desoxyribonuclease B	+		
Desoxyribonuclease C	+		
Ribonuclease	?	Produced by most strains	
Hyaluronidase	+	Produced by most members of types 4 and 22 Found only in minute amounts in culture fluids of most other strains	Production enhanced by presence of substrate
Streptococcal proteinase precursor	+	Produced by majority of strains	Precursor formed only at pH 5 5 to 6 5 Spontaneous activation under reducing conditions
Streptococcal proteinase			
Amylase	?	Variable but produced by many strains	Production enhanced by presence of substrate

atous area of more than 10 mm diameter which appears within 6 to 24 hours

The effects of the toxin are neutralized by antibody A susceptible child shows a positive Dick test and this reactivity persists during the acute phase of scarlet fever However *after convalescence the skin test becomes negative* in the vast majority of patients as the result of antibody formation Inhibition of the effects of toxin by antibody is further demonstrated by the fact that the intradermal injection of potent antitoxic sera will result in blanching of the rash in the early stages of scarlet fever (Schulz and Charlton 1918)

The erythrogenic toxins produced by all strains of group A streptococci are not identical Thus Coffey (1938) in a survey of a large number of strains found that the toxins of about 80 per cent were neutralized by a single antitoxic serum while most of the remaining 20 per cent were neutralized by one or the other of two additional antisera The existence of serologically distinct erythrogenic toxins clarifies some of the irregularities observed in reversal of the Dick test after scarlet fever and also provides a rational explanation for the occurrence of second attacks of the disease

The most prevalent variety of erythrogenic toxin has been obtained in a concentrated and partially purified state some of these preparations having a potency of 10^6 skin test doses per mg N (Stock 1939) The chemical nature of the toxin has not been determined Different strains of streptococci appear to vary in the amount of erythrogenic toxin produced in artificial media although the assay procedure does not allow for accurate quantitative studies However the differences are great enough to suggest that they may play a role in the variability of the occurrence of scarlet fever in different epidemics of streptococcal disease

and R antigens are the most prominent representatives of this class

Hyaluronic Acid Capsule Several independent attempts to demonstrate antigenicity of streptococcal hyaluronate have resulted in failure Despite the apparent nonantigenicity of this substance it is appropriate to consider it with the other surface constituents of the cell because of its peripheral distribution In its native state it is a mucopolysaccharide of high molecular weight which forms highly viscous solutions and is composed of equimolar quantities of N acetyl glucosamine and glucuronic acid It does not appear to be distinguishable chemically from mammalian hyaluronate, a fact which may have some bearing on its lack of antigenicity

As indicated in the discussion of morphology of the streptococcal cell in young cultures the hyaluronate surrounds the organism in a well defined capsular structure As growth proceeds the capsules tend to diminish or disappear and the hyaluronate is found in solution in the medium In general, the environment provided by host tissues appears to promote capsule formation so that the behavior of capsules in vivo may be quite different from that observed in artificial media

The role of the hyaluronate capsule in virulence is not altogether clear but there is some evidence that it may help to protect the organism from phagocytosis and destruction and thus potentiate the antiphagocytic effect of the M protein In the case of mouse infections with encapsulated group C streptococci a marked degree of protection can be achieved by treating infected animals with hyaluronidase With group A strains the effect of hyaluronidase treatment of mouse infection is much less dramatic and protection is observed only when very low infecting doses are employed This is in contrast with the marked protective action of type specific anti M serum (Rothbard 1948) The relative importance of hyaluronate and M protein in natural infections of man has not been determined directly, although here again it appears probable that the M protein plays a dominant role

Extracellular Products of Group A Streptococci

Group A streptococci elaborate a wide variety of biologically active substances including toxins and enzymes, which accumulate in the culture medium during growth Not all of these extracellular products are produced simultaneously by any single strain, but most strains are capable of forming a majority of them It is known that environmental conditions exert a profound effect on the production of certain of the extracellular substances consequently, their occurrence in infected host tissues may differ from that observed in artificial media However, since specific antibodies to most of these substances can be found in the sera of patients convalescing from streptococcal infections it is evident that the organisms retain the ability to produce them under in vivo conditions

The list of the known extracellular products (Table 32) probably falls far short of representing the total capacity of group A streptococci in this regard This is indicated not only by the fact that new substances continue to be discovered but also by the results of serologic and electrophoretic studies of concentrated culture fluids which suggest that the known substances can account for only a fraction of the streptococcal protein present

The extent to which the various extracellular substances contribute to the pathogenicity of hemolytic streptococci can for the most part only be surmised since it is difficult to evaluate the importance of a single enzyme or toxin in the over all interaction between parasite and host tissues However on general biologic grounds it seems likely that they are useful to the survival of the organism in its natural habitat which must be assumed to be human tissues The properties of the extracellular substances are discussed in the following sections

Erythrogenic Toxin Erythrogenic toxin is the substance responsible for the characteristic skin rash of scarlet fever Its mode of action is unknown and the only assay procedure available for the study of the toxin filtrates of streptococcal cultures is a skin test depending on intracutaneous injection of the material in humans (Dick test 1924) or certain susceptible animals The potency of the toxin is such that culture filtrates of appropriate strains can be diluted 100 fold or more and thus the nonspecific irritating effect of other substances in the broth usually can be avoided A positive Dick test is an erythematous and often edem

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Erythrogenic toxin	+	Produced by most strains	Evidence for at least 3 serologically different toxins
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Streptolysin O	+	Produced by most strains	Active in reduced state *only*
Diphosphopyridine *nucleotidase*	+	Production more common *among* some serologic types than others	
Streptokinase	+	Produced by most strains	
Desoxyribonuclease A	+	One or more produced by all strains	Three serologically distinct enzymes which attack the same substrate
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Desoxyribonuclease C	+		
Ribonuclease	?	Produced by most strains	
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Certain strains of group C and group G streptococci have been found to produce a toxin with similar properties

Streptolysin S This agent is responsible for the zones of hemolysis which surround streptococcal colonies on the surface of blood agar plates The designation "S" refers to serum and derives from the fact that originally the hemolysin was thought to be selectively soluble in serum and extractable from living streptococci by serum (Todd 1938) Thus it was observed not only that little streptolysin S is produced during growth unless serum is present in the medium but also that potent preparations could be obtained by shaking washed streptococcal cells in whole serum Subsequent studies have established that this behavior results from the ability of some unidentified constituent of the serum to stimulate production and release of the hemolysin rather than from differential solubility and extraction

An important advance in the study of streptolysin S came from the finding of Okamoto (1939) that yeast ribonucleic acid is highly active in inducing formation of a hemolysin which appears to be identical with the serum induced hemolysin Detailed studies of this material have been made by Okamoto and by Bernheimer (see review by Bernheimer 1954) Ribonucleic acids from a variety of sources but not that from tobacco mosaic virus stimulate the production of large amounts of streptolysin S either when present in culture media in which the organisms are grown or when added under appropriate conditions to washed suspensions of living streptococci The activity of ribonucleic acid is enhanced by digestion with ribonuclease and the active component is associated with the nondialyzable 'core' remaining after enzymatic treatment It seems unlikely that the active component in serum responsible for streptolysin S production can have a similar chemical composition and the relationship between the two kinds of inducing agent is unknown

Partially purified preparations of streptolysin S with potencies as high as 390 000 hemolytic units per mg N have been obtained (Bernheimer, 1954) These preparations contain both protein and polynucleotide However, the activity of the material is destroyed by certain proteolytic enzymes and the hemolytic activity is electrophoretically separable from the polynucleotide so that it seems probable that streptolysin S is protein in nature Despite this fact, the present evidence indicates that streptolysin S is not antigenic, or at least that any antibodies formed are not able to neutralize its hemolytic action Inhibitory antibodies are not found in sera of immunized animals or of patients convalescing from streptococcal disease Earlier studies which suggested the occurrence of neutralizing antibody in low titer have been shown to depend on the nonspecific inhibitory effect of serum lipoprotein Phospholipids in the form of lipoprotein complexes appear to play the major role in this inhibition

Attempts to determine whether streptolysin S has toxic effects other than that represented by hemolysis of erythrocytes have been hampered by the fact that pure preparations are not available so it is not possible to be certain that any pharmacologic or pathologic effect observed is not due to some other substance present in the material At present it must be looked upon as a hemolysin which conceivably may have additional harmful effects on certain host cells or tissues

The production of streptolysin S by groups of streptococci other than A has not been studied adequately although it must be assumed that in most instances an analogous lysin is formed which is responsible for lysis on the surface of blood agar plates No systematic information is available on the effect of ribonucleic acid on the production of hemolysin by other groups

Streptolysin O This hemolysin is so designated because of its oxygen lability and is quite distinct from streptolysin S (see summary of comparative properties in Table 33) It is hemolytically inactive in the oxidized form but is readily activated by the addition of reducing agents such as sulfhydryl compounds This property of reversible oxygen lability is shared with certain other bacterial hemolysins e g pneumolysin and tetanolysin It is responsible for the failure of streptolysin O to participate in the formation of hemolytic zones around colonies of streptococci grown aerobically on the surface of blood agar The reducing conditions which develop in the environment of deep colonies in blood agar pour plates are sufficient to activate the

TABLE 33 DIFFERENCES IN PROPERTIES OF STREPTOLYSIN O AND S*

	STREPTOLYSIN O	STREPTOLYSIN S
Activation by SH compounds	+	—
Neutralization by specific antibody	+	—
Formation stimulated by serum or by polyribonucleotide	—	+
Trypsin sensitivity	+	—
Inhibition by low concentrations of cholesterol	+	—
Inhibition by low concentrations of lecithin	—	+
Rate of lysis	Not directly proportional to lysin concentration	Directly proportional to lysin concentration
Induction period prior to lysis	Short	Long

* Adapted from Bernheimer 1954

hemolysin Streptolysin O is produced by almost all strains of group A streptococci and strains which lack this property are encountered only rarely It is also formed by many strains of groups C and G

Streptolysin O has not been obtained in pure form but since it is readily destroyed by proteolytic enzymes it would appear to be protein in nature It is antigenic eliciting the formation of antibodies which effectively neutralize its hemolytic action A high proportion of patients with streptococcal infection show an antibody response during convalescence consequently the measurement of serum antistreptolysin O has become widely used as a test for establishing the occurrence of a recent streptococcal infection Both the oxidized and the reduced forms will combine with specific antibody indicating that reversible oxidation is not associated with major changes in the antigenic properties of streptolysin O Cholesterol is highly active as an inhibitor of streptolysin O For some reason the free cholesterol of normal serum does not exert an appreciable inhibitory effect and does not interfere with the measurement of specific antistreptolysin O However bacterially contaminated serum or serum subjected to certain kinds of chemical treatment may develop inhibitory activity as a result of changes in the lipoproteins

Despite the fact that streptolysin O has not been obtained in pure form study of its biologic effects have met with more success than in the case of streptolysin S This is made possible by the availability of three specific and unrelated technics for inhibiting its biologic activity oxidation cholesterol inhibition and inhibition by specific antibody Thus any property of a streptolysin O preparation which is nullified by all three technics can reasonably be assumed to be due to the hemolysin Using these technics Todd (1942) has presented evidence that streptolysin O is toxic for leukocytes It has been shown to act as a lethal toxin on intravenous injection into a variety of laboratory animals and one possible explanation for its lethal action has come from studies of its cardiotoxicity Bernheimer and Cantoni (1945) first demonstrated that streptolysin O in small amounts causes systolic standstill in the isolated frog heart More recently these studies (Kellner et al 1956) have been extended to a study of the mammalian heart The isolated hearts of rat rabbit and guinea pig all respond with irreversible loss of myocardial contractility when as little as 25 to 50 hemolytic units of streptolysin O are added to the perfusion fluid Partially purified preparations of the hemolysin have been obtained with a potency as high as 33 000 hemolytic units per mg N and thus it is apparent that very minute amounts are sufficient to exert a cardiotoxic effect

Diphosphopyridine Nucleotidase (DPNase) Streptococcal DPNase one of the most recent additions to the list of extracellular substances was discovered in the course of attempts to determine the mechanism of the cardiotoxic effect of streptolysin O on mammalian hearts The streptolysin O preparations used were found to inhibit certain of the metabolic enzymes of mammalian heart muscle (Carlson et al 1956) but this enzyme inhibition was not affected by any of the procedures known

to inactivate the hemolysin Thus it was nec essary to attribute the effect to some unknown component of the preparation The key to its nature was provided by the fact that all of the enzyme systems inhibited involve the co enzyme DPN, and direct test revealed the presence of a highly active DPNase which acts by liberating nicotinamide from the mole cule (Carlson et al 1957)

Streptococcal DPNase is produced by a wide variety of group A strains, although many are encountered which do not form ap preciable amounts even when grown in the presence of serum a procedure which increases the yield of the enzyme There appears to be some relationship between DPNase production and serologic type For example, Lazarides and Bernheimer (1957) have found that all of 23 strains of type 3 and 52 of 58 strains of type 12 produce the enzyme while all of 33 strains of type 1 and 38 strains of type 19 do not Among other serologic groups certain strains of group C and G possess this property

The enzyme is antigenic and antibodies which inhibit its activity are found in the serum of patients convalescing from strepto coccal infections A possible toxic role for DPNase has been suggested as a result of studies of the phenomenon of leukotoxicity This phenomenon first described by Levaditi (1918) years ago is manifested by sudden death and disintegration of leukocytes after the ingestion of streptococci Wilson (1957) has shown that certain streptococcal strains consistently exert this effect on a proportion of the leukocytes which phagocyte them while others apparently lack the ability to injure the leukocyte In a collaborative study (Bern heimer Lazarides and Wilson 1957) involving 39 different streptococcal strains it has been found that there is an excellent correlation between the ability of a strain to produce DPNase and its leukotoxicity All but 2 of the DPNase positive and none of the DPNase negative strains proved to be leukotoxic While these findings suggest that the enzyme may be concerned in the destruction of the leukocyte some form of direct confirmatory evidence will be needed to establish the rela tionship

Streptokinase The occurrence of a sub stance in streptococcal culture filtrates which promotes the lysis of human fibrin clots was first described by Tillett and Garner (1933), and the active agent was termed streptococcal fibrinolysin Subsequently, it was found (Mil stone, 1941) that fibrinolysis depends upon the presence of an essential serum factor which was later identified as the inactive precursor of a proteolytic enzyme (Christensen 1945) The accumulated evidence indicates that the streptococcal substance exerts its effect by causing the activation of this precursor, termed plasminogen, and because of the similarity to the activation of trypsinogen by enterokinase, the term streptokinase was suggested as more appropriate than fibrinolysin Recent findings have raised the possibility that streptokinase does not act directly on plasminogen but rather on a proactivator which in turn acti vates plasminogen This human plasma prote ase system is further complicated by the oc currence of natural inhibitors which affect both the activation process and the enzymatic activity of the protease

Streptokinase is a remarkably potent acti vator of the human protease system Partially purified preparations, containing 2 000 fibrino lytic units per microgram of nitrogen have been obtained by Christensen (1954) who estimates that as little as 0 00005 microgram of streptokinase nitrogen will exert a detect able effect on a fibrin clot In general it ap pears to be much less effective in the protease systems of other animal species This may depend on both quantitative and qualitative differences in plasminogen and inhibitors

The biologic activity is readily destroyed by trypsin, suggesting that streptokinase is protein in nature It is antigenic, and again the antibodies formed specifically inhibit its action The inhibitory effect of antibody can be clearly distinguished from the effects of natural inhibitors of the protease system and methods have been devised for measuring the amount of antibody in human sera Increases in titer of specific antistreptokinase occur in a high percentage (70 to 80%) of patients fol lowing streptococcal infections

Strains of group A streptococci vary over a wide range in the amount of streptokinase pro duced when the organisms are grown under favorable conditions in artificial media but very few strains appear to lack this property completely Certain group C strains elaborate streptokinase in large amounts and these have

been used in large scale production of the substance which has been undertaken because of the therapeutic value of its fibrinolytic action It is also produced by some group G strains

The possible part that streptokinase plays in the pathogenesis of streptococcal infection is unknown and there is no clear indication that activation of the plasma proteolytic enzyme results in toxic effects or tissue damage However it seems inescapable that streptokinase must prevent the formation of an effective fibrin barrier and thus influence the character of lesions by interfering with localization of the infection

Desoxyribonuclease An enzyme which depolymerizes desoxyribonucleic acid appears in the culture fluid during growth of streptococci (Tillett et al 1948 McCarty 1948) Initial studies of the enzyme indicated that it is analogous to pancreatic desoxyribonuclease in that it requires activation by magnesium ions and has its greatest activity between pH 7 and pH 8 5 Desoxyribonuclease is produced by all group A strains in readily detectable amounts and is also formed by representatives of several other groups As in the case of many of the other extracellular streptococcal products strains of groups C and G are prominent among the latter

Antibodies to desoxyribonuclease have been obtained by injecting rabbits with a purified preparation of the enzyme from a group A strain and similar antibodies occur in the serum of patients after streptococcal infections (McCarty 1949) In both cases the antibodies inhibit the action of the enzyme on its substrate However the incidence of antibody production in patients was found to be quite low (less than 40%) despite the fact that all group A strains produce the enzyme in vitro

The knowledge of streptococcal desoxyribonuclease has been significantly advanced by the recent studies of Wannamaker (1958) which also provide an explanation for the low antibody response that was originally observed in human patients Group A streptococci have been shown to produce 3 serologically distinct desoxyribonucleases designated A B and C The 3 enzymes have been separated by zone electrophoresis of concentrated preparations from culture fluids and studied individually Each of the 3 is magnesium activated but they show differences in their pH of optimum activity Antibodies produced in rabbits specifically inhibit the homologous enzyme in each case and do not affect the activity of the other 2 enzymes Serum from patients with rheumatic fever or convalescent from streptococcal infections also show marked differences in their ability to inhibit the 3 enzymes A high percentage of patients comparable with that observed in the case of antistreptolysin O produce inhibitory antibody to desoxyribonuclease B while the response to A and C appears to be inconstant and irregular

Strains of group A streptococci vary in the relative amounts of the 3 enzymes elaborated An insufficient number of strains have been examined to determine whether certain patterns exist but it is clear that some strains produce all 3 enzymes The serologic studies with human sera suggest that desoxyribonuclease B is either the most common or the most antigenic of the 3 The organism used in the preparation of enzyme for the original serologic studies (McCarty 1949) has been shown to produce predominantly desoxyribonuclease A and this accounts for the low incidence of antibody responses observed in patients No information is available concerning the possible diversity of group C and G desoxyribonucleases and desoxyribonuclease A was predominant in the one group C strain examined

The production by streptococci of 3 enzymes which attack the same substrate is of theoretic interest It is possible that each enzyme hydrolyzes a different specific linkage in the substrate macromolecule but comparative studies of the split products have not yet been made There is no evidence that desoxyribonuclease attacks living cells and thus it is unlikely that the streptococcal enzymes exert a deleterious effect on tissues during streptococcal infections However they are effective in hydrolyzing the nucleic acids and the nucleoproteins released by necrotic cells and the split products are apparently utilized by the micro-organism indicating that the enzymes may have nutritional significance The effect of desoxyribonuclease on nucleoproteins has been exploited as a therapeutic tool in the liquefaction of highly viscous exudates which result from the disintegration of leukocytes as in the case of empyema Preparations containing streptokinase and desoxyribonuclease

(streptodornase) from group C strains are commercially available and have been employed in a variety of conditions in which *fibrinous* or *nucleoprotein* containing exudates accumulate (Tillett, 1950)

Ribonuclease has also been found in culture fluids of group A streptococci (McCarty, 1948) However, it occurs in relatively small amounts and no information has been obtained concerning its antigenicity or the presence of specific antibody in human sera

Hyaluronidase The production of an extracellular hyaluronidase by an organism which is also able to elaborate a hyaluronic acid *capsule would appear at first glance to present* something of a paradox especially since it *is known that the capsule is destroyed* readily by the enzyme However, in vitro studies indicate that streptococci rarely produce both substances simultaneously Therefore the two functions are to some extent mutually exclusive although there is reason to believe that a single strain may produce capsules while growing under one set of conditions and hyaluronidase under different conditions Hyaluronidase *accumulates in relatively large* amounts in the culture fluids of most strains of type 4 and type 22 (Crowley, 1944) and these strains are apparently never encapsulated *Only rare strains of other types have* been described as producing comparable amounts of hyaluronidase In the case of the majority of group A strains the presence of an extracellular hyaluronidase can be demonstrated if at all only by highly sensitive tests and often after prolonged incubation even with the use of these technics the amount produced is extremely small compared with that of type 4 and 22 strains The production of the enzyme by type 4 and 22 strains is enhanced by the addition of hyaluronate to the medium (Rogers 1945)

The results of antibody studies suggest that the in vitro findings do not reflect the true potentialities of group A streptococci with respect to hyaluronidase formation Following streptococcal infections a high percentage of patients show an increasing titer of antibodies which *specifically inhibit* hyaluronidase preparations obtained from type 4 or 22 strains It has been established that this is a true antibody and is not related to the nonspecific hyaluronidase *inhibitors found in* normal sera Since these antibodies are formed regardless of the type of streptococcus associated with the infection, it seems obvious that the pattern of hyaluronidase production observed in laboratory studies is misleading and that most group A strains must produce hyaluronidase during infections in sufficient amounts to stimulate antibody production The explanation of this discrepancy remains obscure, and it may involve the action of some undefined factor *in the in vivo environment that stimulates* production or activation of the enzyme

Streptococcal hyaluronidase like other hyaluronidases has its optimal activity in the acid range (pH 6 0 or below) and in the presence of NaCl or other salts The effect of salt may be largely the result of prevention of inhibition by impurities in the substrate rather than activation of the enzyme A variety of technics are used in measurement of enzyme activity Chemical methods, such as estimation of reducing substances released during digestion are relatively insensitive and have not been employed as extensively as physical methods The latter depend on changes in the properties of the substrate which result from splitting the macromolecule into smaller units for example, fall in viscosity of solutions (viscosimetric test) or loss of the ability to form insoluble complexes with proteins after acidification (turbidimetric and mucin *clot* prevention tests) The mucin clot prevention test although the least precise and quantitative of the several methods is readily adaptable for *the measurement of antibody by the serial* dilution technic and has been widely used for this purpose The hyaluronic acid substrate for all of these methods is usually prepared from *human umbilical cords* but some difficulty has been encountered in obtaining reproducible and standardized hyaluronate preparations

Streptococcal hyaluronidase differs from testicular hyaluronidase in several respects The end products of hydrolysis by the bacterial enzyme are not identical with those of the mammalian enzyme and the former does not attack chondroitin sulfate

Hyaluronidase production occurs in streptococci other than those of group A notably in certain strains of groups B C and G In the case of groups C and G it has been shown that the enzymes are serologically distinct from those of group A and are not significantly inhibited by antibody to the group A enzyme

Streptococcal Proteinase This enzyme and its inactive precursor are the only extracellular streptococcal substances which have been obtained in crystalline form (Elliott 1950) They are also unique by virtue of the marked effect exerted by environmental conditions on their production Proteinase precursor is released by streptococci only when the pH of the medium is maintained between 5 5 and 6 5 Thus none of this substance is formed under the optimal conditions for growth of the organisms between pH 7 0 and pH 8 0 which also favor the production of the other extracellular enzymes As in the case of streptolysin S and desoxyribonuclease precursor is formed by washed streptococcal cells when suspended in an incomplete medium Salts and glucose are sufficient for this purpose if the pH is maintained below 6 5 but production is greatly enhanced by the addition of peptone products The capacity of streptococci to produce this substance under appropriate conditions appears to be much greater than for the other extracellular enzymes and Elliott (1950) reports the recovery of 169 mg of 6 times recrystallized material from 10 liters of culture filtrate

The precursor is autocatalytically converted to active proteinase under suitable reducing conditions (Elliott and Dole 1947) This conversion may occur in culture media if aerobic conditions do not prevent a fall in oxidation reduction potential and can be achieved in solutions of the crystalline precursor by the addition of sulfhydryl compounds or other reducing agents The autocatalytic effect appears to depend on the initial presence of traces of active enzyme The action of trypsin in low concentration also brings about rapid conversion of the precursor Formation of active enzyme by either method is accompanied by the release of dialyzable split products amounting to 40 per cent of the weight of crystalline precursor Streptococcal proteinase resembles cathepsins and plant enzymes of the papain family in requiring activation by sulfhydryl compounds or comparable reducing agents Studies on synthetic substrates indicate that it has an unusually broad substrate specificity (Mycek et al 1952)

The production of proteinase by streptococci appears to be largely limited to members of group A It has been found in representative cultures of most of the serologic types, although enhancement of virulence by mouse passage results in suppression of its production (Elliott 1945) Growth of streptococci under conditions which allow the appearance of active proteinase in the medium can have a profound effect on other biologic properties Thus the M protein is destroyed because of its great susceptibility to proteolytic digestion and serologic typing of the organisms becomes impossible (Elliott 1945) Furthermore certain of the other extracellular products e g streptokinase and hyaluronidase may be destroyed by proteolytic action

Precipitating antibodies have been prepared in rabbits against both crystalline proteins The proteins differ serologically and the precursor behaves as though it has two antigenic components one of which is specific and the other identical with the proteinase antigen (Elliott 1950) Rabbit and horse antibodies to proteinase have been shown to inhibit the action of the enzyme However inhibitory antibodies are not readily demonstrated in sera of patients after streptococcal infection and the titers observed are so low that the possible role of non pecific proteinase inhibitors cannot be eliminated (Todd 1947)

It is not known whether or not streptococcal proteinase causes tissue damage in the course of streptococcal infections The requirement of low pH and reducing conditions for the release of the enzyme suggests that it will be formed only in certain kinds of infection as for example in an abscess where the appropriate environment would be expected That the enzyme is capable of inducing tissue changes in experimental animals has been demonstrated by Kellner and Robertson (1954) who found that intravenous injection of activated streptococcal proteinase results in the formation of necrotic myocardial lesions in rabbits guinea pigs and mice This effect is also shown by other proteolytic enzymes and thus is not specific for streptococcal proteinase

Amylase Many strains of group A streptococci elaborate an extracellular amylase when grown in the customary peptone media (Crowley 1950 1954) The enzyme has the properties of an α amylase and hydrolyzes glycogen and amylopectin in addition to starch Production of amylase varies greatly among strains and even individual members of a

population of a single strain may show differences in enzyme activity The presence of substrate in the medium greatly enhances the amount of amylase released

Further studies have revealed that the same strains which produce amylase will, under different cultural conditions, synthesize a starch like polysaccharide (Crowley 1955 Crowley and Jevons 1955) The presence of human plasma in the medium is important for the formation of the substance and maltose serves as an adequate substrate although starch and glycogen are also effective These findings provide another example of the marked influence of environmental conditions on the biologic behavior of streptococci

The antigenicity of amylase and the possible occurrence of antibodies in human sera have not been investigated

Intracellular Components

The intracellular components of group A streptococci have received little attention in comparison with that given to the surface constituents and extracellular products In part this is referable to the fact that cellular extracts represent highly complex mixtures which are difficult to resolve Such extracts contain a variety of proteins including the various metabolic and synthetic enzymes of the cell and large amounts of other material such as nucleotides and nucleic acids A nucleoprotein fraction, obtained by extraction with weak alkali has been examined for its serologic properties (Lancefield 1925) Although this material probably makes up a large portion of the total cellular contents and must be very heterogeneous antigenically the serologic studies are useful in indicating interrelationships between bacterial species Thus nucleoproteins from hemolytic streptococci react with antisera from animals immunized with nucleoproteins from both hemolytic and nonhemolytic streptococci as well as with similar preparations obtained from pneumococci A lesser degree of cross reactivity was observed with antisera to staphylococcal nucleoproteins

The properties of individual cellular enzymes have been investigated in only a few instances Jacox (1953) reported the occurrence of a β glucuronidase which he found in representatives of only 4 of 32 different types of group A streptococci There was no evidence of inhibition of the enzyme by the sera of patients convalescing from streptococcal infections The lipoproteinase described by Krumwiede (1954) which causes opalescence in serum by splitting α_1 lipoprotein probably represents a cellular enzyme, since it is extractable from washed organisms with 40 per cent urea However it may be released into the environment under conditions reminiscent of those which affect the production of streptolysin S that is, it is found in serum broth cultures and appears to be extractable from cells with serum Preliminary studies suggest that certain human sera may contain antibodies which inhibit lipoproteinase, and inhibitory antisera have been obtained by immunizing rabbits with crude enzyme preparations

Recent attempts have been made to resolve the cellular components of group A streptococci into definable fractions by electrophoretic technics (Hess and Slade, 1955) These experiments involve extracts obtained after disintegration of the cells by sonic oscillation or shaking with glass beads The electrophoretic patterns obtained with extracts of various types have been compared but there is still little information on the biologic activity of the several electrophoretic components

RELATIONSHIP BETWEEN STREPTOCOCCI OF GROUP A AND OTHER GROUPS

The basic similarity in the monosaccharide composition of the cell wall carbohydrates of groups A through G has already been mentioned There is in addition evidence for a close biologic relationship between groups A C and G which is based largely on the study of strains of human origin For example it is evident from the discussion of the extracellular products of group A streptococci that the same or similar substances are in most cases also produced by some strains of groups C and G Certain of the enzymes, such as streptokinase and desoxyribonuclease may be found in cultures of some other groups but it is only in groups C and G that one finds a total complement of extracellular substances approaching that of group A Further support for the interrelationship comes from studies on the distribution of surface protein antigens Thus R antigen serologically identical or

closely related to the group A substance has been found in members of groups C and G streptococci as well as in some of group B (Maxted 1949)

Several of the streptococcal groups other than A have been subdivided into serologic types but in no case has a type specific protein antigen been identified which has been *clearly shown to have a relationship to viru*lence comparable with that of M protein Type differentiation in group C streptococci has not been explored intensively but it appears to depend on surface protein antigens and it is possible that in some instances these may be M like in their biologic properties In the case of other groups in which specific types have been described (groups B D F and G) the available evidence suggests that the type specific substance is polysaccharide in nature This has been clearly established in the case of group B streptococci which have polysaccharide capsules analogous to those of pneumococci

The various similarities and dissimilarities between hemolytic streptococci do not fall in sufficiently uniform patterns to allow for many broad generalizations On the whole however it seems clear that they are much more closely *related to one another than to the numerous* nonhemolytic and anaerobic streptococci

HEMOLYTIC STREPTOCOCCAL INFECTIONS IN MAN

Hemolytic streptococci are associated with a wide variety of disease entities in man and *in the vast majority of these acute infections* the causative organisms belong to group A Streptococci of groups C and G possibly as a reflection of their biologic similarities to group A are sometimes implicated in similar infections especially those involving the upper respiratory tract These infections with groups C and G streptococci are characteristically mild and usually do not lead to the complications observed in group A infections They are not known to initiate late sequelae such as rheumatic fever or glomerulonephritis Occasionally other groups of streptococci are encountered in association with pathologic processes which are more commonly caused by group A streptococci For example puerperal fever can result from group B streptococcal infection of the postpartum endometrium and septicemia of the newborn can be caused by these organisms

Hemolytic streptococcal infections are to be distinguished from infections associated with the various nonhemolytic streptococci which are found among the normal flora of the respiratory and the gastro-intestinal tracts These organisms frequently find their way *into the blood stream in small numbers but* under ordinary conditions they are readily disposed of by the natural defense mechanisms However in the presence of anomalies of anatomic structure they may become established as infectious agents Thus subacute bacterial endocarditis which affects heart valves altered by congenital malformations or rheumatic heart disease is most frequently associated with this class of organisms Similarly infections of the urinary tract with nonhemolytic streptococci may be encountered when anomalies or obstruction of the outflow tract are present The nature of the organism which lodges on the heart valves in endocarditis would appear to be a matter of chance rather than the expression of specific pathogenic properties Although the streptococci involved usually do not belong to one of the recognized groups members of certain serologic groups are also encountered For example group D streptococci which normally inhabit the gastro intestinal tract (enterococci) are often incriminated in endocarditis as well as in urinary tract infection Members of other groups (e g group O) are also occasionally isolated from the blood stream of *patients with endocarditis*

Because of the pre eminent importance of group A streptococci in human disease the following sections deal primarily with the characteristics of infections with these organisms

RESPIRATORY TRACT

The primary site of invasion of the human body by group A streptococci is through the upper respiratory tract Streptococcal pharyngitis or tonsillitis is by far the most common of all streptococcal infections and provides the focus from which the organisms are disseminated to initiate most other forms of streptococcal disease

Streptococci appear to have a special predilection for the lymphatic system and in the upper respiratory tract the initial localization

of the infection is in the lymphoid tissue of the pharynx The infectious process is manifested by swelling and reddening of the tonsils and other pharyngeal lymphoid tissue, and in the classic disease is accompanied by the appearance of focal or confluent accumulations of exudate on the affected areas The adjacent mucous membranes are also commonly involved in the inflammatory process These changes cause soreness of the throat which may be quite marked, and in addition the infection leads to systemic manifestations such as fever and general toxicity While this represents the typical picture of the full blown disease streptococcal pharyngitis can also occur with only minimal evidence of inflammation and minimal symptoms

Complications of this relatively superficial infection occur by extension either through lymphatic channels or by direct involvement of other areas The cervical lymph nodes at the angle of the jaw which drain the tonsillar area are usually involved to some degree and become swollen and tender In severe cases infection of these nodes may progress to the formation of purulent abscesses Abscesses may also form in the deep peritonsillar tissues (quinsy) and particularly in infants in the retropharyngeal tissues Like many of the other severe manifestations of streptococcal disease peritonsillar and retropharyngeal abscesses are now relatively rare This is true also of Ludwig s angina which represents cellulitis and subsequent abscess formation in the tissues of the neck especially at the base of the tongue and on the floor of the mouth The extensive swelling associated with all three of these conditions often leads to difficulty in swallowing and serious embarrassment of respiration

Streptococcal infections of the pharynx readily extend to the paranasal sinuses through the natural openings Similarly the eustachian tube is frequently involved and leads to acute infections of the middle ear Both the sinusitis and the otitis media which result from these extensions may develop into chronic purulent processes In the case of otitis media the infection can invade the air cells of the mastoid bone causing mastoiditis and ultimately osteomyelitis of the surrounding bony structures If invasion of the bone progresses to the meningeal surfaces, serious complications such as meningitis or cerebral sinus thrombosis may result

Extension of streptococcal pharyngitis may also occur downward into the lower respiratory tract Here it gives rise to bronchitis and interstitial bronchopneumonia characterized by extensive involvement of the lymphatic vessels Spread of the infection by the lymphatics, typical of the streptococcal lymphangitis in other areas follows the flow to the draining lymph nodes but may proceed in a retrograde direction in occluded vessels and reach the pleural surfaces Because of this retrograde extension, pleurisy is common in streptococcal pneumonia and leads to the formation of large quantities of serofibrinous pleural exudate

Relative to the great frequency of upper respiratory infections with streptococci pneumonia is a rare event Certain viral infections of the respiratory tract seem to promote the occurrence of this complication when they occur simultaneously with streptococcal infection During World War I for example a large number of cases of streptococcal pneumonia with massive pleural effusion were encountered among military personnel in the course of measles and in influenza epidemics

Scarlet Fever

Scarlet fever was recognized as one of the common exanthematous diseases of children for centuries before its streptococcal etiology was discovered Because of this history it is usually considered as a separate disease entity although modern bacteriologic findings indicate that it merely represents streptococcal pharyngitis or tonsillitis with an accompanying rash Thus in a given epidemic of streptococcal disease a single predominant type of group A streptococcus may be recovered from cases of both scarlet fever and streptococcal sore throat

The erythrogenic toxin is responsible for the rash of scarlet fever but the importance of this substance in other toxic manifestations of the disease has not been clearly established As indicated in the discussion of the extracellular products of group A streptococci this substance is only one of several with potentially toxic properties Symptoms of systemic toxicity occur in the case of streptococcal sore throat which are indistinguishable from those

of scarlet fever and the degree of this manifestation is more a function of the severity of the disease process than of the presence or the absence of a skin rash Other clinical findings of scarlet fever such as the so-called strawberry tongue resulting from the appearance of prominent papillae on an otherwise smooth and diffusely reddened organ also occur in uncomplicated streptococcal pharyngitis The purulent complications of scarlet fever (cervical adenitis sinusitis otitis etc) are identical with those described for streptococcal pharyngitis and the same delayed nonsuppurative sequelae are encountered

The rash of scarlet fever is characterized by diffuse reddening of the skin which may cover most of the body but is frequently most prominent on the trunk The changes induced in the skin result in desquamation during convalescence This is especially evident on the hands and the feet where a thick keratinized layer is present

There are certain anomalies in the occurrence of scarlet fever which are not satisfactorily explained on the basis of susceptibility or immunity to a primary toxin For example the disease is rarely seen in infants and very young children and infants do not give positive reactions with Dick toxin These findings cannot be correlated with the transfer of passive immunity from the mother and have led to the suggestion that the development of hypersensitivity through prior exposure to erythrogenic toxin is required before this agent can induce the formation of the characteristic rash In any event it is clear that immunity to the toxin is effective in preventing second attacks of the scarlet fever in the vast majority of cases although it has no effect on the occurrence of subsequent streptococcal infections As noted earlier the occasional occurrence of second attacks of scarlet fever can be explained by the fact that certain strains of group A streptococci produce an erythrogenic toxin which is serologically distinct from that of the majority of strains It is evident also that some epidemics of streptococcal disease are much more commonly associated with scarlet fever than others This may conceivably be referable to the relative capacity of individual strains to produce erythrogenic toxin in large amounts in vivo but a correlation of this kind has not been demonstrated by laboratory studies

Scarlet fever has been described in association with upper respiratory infections with group C streptococci and even in infections with more distantly related organisms the staphylococci In the latter case the staphylococci involved are found to produce an erythrogenic toxin with properties like that of the streptococcal toxin However these are relatively rare exceptions and in nearly all cases of the disease group A streptococci are recovered on culture

Other Acute Streptococcal Infections

While the upper respiratory tract is the principal site of group A streptococcal infections these organisms have the capacity to localize and produce infections in many other areas The initiation of infection in these areas may result either from hematogenous spread or from the chance inoculation of susceptible tissues with organisms from the environment The adaptability of streptococci to the invasion of human tissues is well illustrated by their behavior when introduced directly through the normal skin barrier This can occur in a variety of accidental injuries as in the case of infection of cuts or blisters or in natural portals of entry such as the umbilical cord of the newborn infant The course of events following production of lesions of this type by virulent streptococci in the extremities follows a well defined pattern The lymphatic vessels are quickly involved often with minimal evidence of reaction at the site of the local lesion and the occurrence of advancing lymphangitis is visibly manifested by the appearance of red streaks moving up the arm or the leg The draining lymph nodes become swollen and tender but do not significantly check the centripetal spread of the lymphatic infection which continues until it reaches the blood stream and gives rise to septicemia Frequently the progress of this type of infection is so rapid that septicemia and death can occur within 24 to 48 hours of the initial injury Variations in virulence of the organism or in host resistance may alter this course of events and one may even encounter localized purulent infections of the skin which spread only in an indolent fashion

Septicemia may develop as a complication

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pathologic finding is a focal inflammatory process characterized by the occurrence of unique myocardial lesions known as Aschoff bodies Involvement of the endocardium can result in deformative scarring of the valves and adjacent structures which leads to chronic rheumatic valvular heart disease

The generalized nature of the rheumatic process is illustrated by the variety of other manifestations which may occur in the course of the disease including involvement of the skin and of the central nervous system (chorea) The disease may become subacute or chronic and in extreme cases evidence of rheumatic activity may persist for years After recovery from rheumatic fever the patient is highly susceptible to recurrence of the disease following a new streptococcal infection and many attacks can occur in a single individual

There appears to be no strain selectivity among group A streptococci in their ability to initiate rheumatic fever since practically all of the prevalent serologic types have been found to be associated with the disease However only a small percentage of patients with streptococcal sore throat develop this late complication and it is evident that host factors must play a role in the pathogenesis of the disease

Acute hemorrhagic glomerulonephritis resembles rheumatic fever in the occurrence of a latent interval between the streptococcal infection and onset of the disease although in this case the average length of the interval is probably shorter As the name suggests this disease involves primarily the glomerulus of the kidney and is associated with the appearance of blood in the urine and general manifestations of kidney disease such as edema and hypertension As might be expected from their common origin rheumatic fever and glomerulonephritis occasionally occur simultaneously in the same patient However there is less evidence of permanent damage in glomerulonephritis and the great majority of recovered patients do not show evidence of chronic kidney damage Furthermore recurrent attacks of glomerulonephritis are rare

An additional important difference in the pathogenesis of the two diseases has been established recently by the finding that only certain types of group A streptococci appear to be associated with the initiation of glomerulonephritis (Rammelkamp and Weaver 1953) These studies now adequately confirmed show that the great majority of cases are preceded by type 12 streptococcal infections and that the remaining cases are usually attributable to one or two additional types This strongly suggests that some specific property of certain strains of group A streptococci is important in the etiology of glomerulonephritis These findings also serve to explain the rarity of second attacks of the disease as contrasted with the frequent recurrence of rheumatic fever

Erythema nodosum the third representative of this group of diseases is less clearly delineated and only a portion of the cases showing the clinical picture of the disease can be attributed to a preceding streptococcal infection However when this relationship exists it follows the same pattern of delayed appearance after the bacterial infection and can recur following subsequent attacks of streptococcal sore throat The disease is manifested by the occurrence of red extremely tender nodular swellings particularly on the extensor surfaces of the extremities in association with fever and signs of general toxicity Lesions of the same type may be encountered occasionally in rheumatic fever but erythema nodosum is seen more commonly in the absence of other rheumatic stigmata and recovery occurs without residual damage

There is evidence to suggest that these late sequelae of streptococcal infection may represent a severe and exaggerated form of a type of host reaction that is commonly manifested during convalescence Thus during the period following apparent recovery from the acute infections one may encounter recurrent swelling and tenderness of the cervical lymph nodes transient arthralgias transient electrocardiographic changes microscopic hematuria etc The time of appearance of these changes as well as the time of onset of the overt poststreptococcal diseases coincides with the time at which antibody response to streptococcal antigens is reaching its maximum and this has been an important factor in the formulation of the theory that hypersensitivity reactions are concerned with pathogenesis of the delayed sequelae

of other localized streptococcal infections It is always a most serious manifestation, accompanied by high fever and severe toxic symptoms Prior to the discovery of effective chemotherapeutic agents the occurrence of septicemia was always a grave prognostic sign In contrast with this form of blood stream infection which is always associated with some focus that provides a continuing supply of organisms transient bacteremia can occur without giving rise to apparent symptomatology unless the organisms are able to establish themselves at another site This phenomenon is responsible for hematogenous dissemination of streptococcal infection Thus, septic arthritis manifested by the accumulation of purulent infected exudate within the joint cavity must be assumed to originate by this process Similarly streptococcal meningitis sometimes appears in the absence of demonstrable mastoiditis suggesting that it originates by way of the blood stream rather than by direct contiguity

The classic puerperal fever in its most virulent form represents a group A streptococcal infection of the postpartum endometrium with subsequent septicemia The evidence indicates that this endometritis results from direct infection of a highly susceptible tissue At the time that this infection was widely prevalent, it was noted that its greatest incidence coincided with epidemics of scarlet fever and streptococcal sore throat increasing the opportunity for dissemination of streptococci from the respiratory tracts of carriers who came into contact with the patient The institution of suitable aseptic technics did much to reduce the danger of puerperal fever even as it reduced the occurrence of streptococcal infection of surgical wounds

Erysipelas is a streptococcal skin infection which, like scarlet fever was originally thought to be a specific disease until it was demonstrated that the organisms responsible belong to the same serologic types as those causing other streptococcal infections This infection characteristically spreads in all directions in the subepidermal tissues from an original focus which may or may not be readily apparent The inflammatory process is manifested by redness and edema and the advancing elevated margin of the lesion is clearly demarcated from normal skin Streptococci are usually demonstrable only in the edema fluid from the advancing edge The area of skin involved may be extensive and in severe cases deeper penetration or septicemia can ensue

Streptococci are also involved in the superficial purulent skin lesions of impetigo contagiosa Here they are usually found in association with staphylococci and the lesions assume a more chronic and indolent character They begin as individual vesicles which subsequently break with the formation of encrusted purulent lesions

Delayed Sequelae of Streptococcal Infections

This group of diseases includes acute rheumatic fever acute hemorrhagic glomerulonephritis and erythema nodosum They represent an entirely different order of disease phenomena from the various suppurative complications of acute streptococcal disease in which the infecting organisms play so prominent a role The mechanisms involved in the initiation of the delayed sequelae by streptococcal infection have not been clarified but it is evident not only that the clinical findings are unique but also that the nature of the pathologic lesions is distinct from that of the processes in which direct bacterial involvement is evident

Rheumatic fever is the most important and widespread of the late sequelae Characteristically this disease is preceded by a typical streptococcal infection of the upper respiratory tract and has its onset after a latent interval which is variable in length with an average of about 3 weeks The nature and the severity of the symptoms of rheumatic fever cover a wide range However the most prominent features of the classic disease are fever migratory polyarthritis and carditis The arthritis is not to be confused with the acute septic arthritis mentioned previously since streptococci are not demonstrable in the joint fluid and recovery occurs without residual damage to the joint Involvement of the heart is the most significant aspect of the disease from the point of view of both the severity of the acute illness and the production of permanent damage All layers of the heart may be affected and signs of myocarditis and pericarditis are frequently present The basic

pearance of detectable levels of M antibody after infection is usually greatly delayed in comparison with antibodies to the extracellular products This can be explained best by assuming that the latter represent the secondary or anamnestic type of antibody response while the M protein is a new antigen to which the individual has not been exposed previously If this explanation is correct it supports the view that the antistreptolysin O type of response characteristic of the older patient is conditioned by prior exposure to the antigen

During the past several decades there seems to have been a progressive decline in the severity of streptococcal infections This is most evident in the case of readily recognizable manifestations such as scarlet fever This decrease in severity unaccompanied by a comparable decrease in the morbidity rate antedates the introduction of antibacterial drugs and it must be assumed that either changes in the virulence of the organism or in the susceptibility of the human population or possibly a combination of both factors are responsible Similar changes in the severity of scarlet fever were reported in past centuries and although the validity of these older observations is uncertain the possibility of recurring cycles of varying disease intensity is suggested The fact that the immunity status of a population can play a key role in the severity of streptococcal disease is indicated by the highly fatal streptococcal epidemics that are seen occasionally in isolated groups not previously exposed to streptococci It is evident that the matter of nonspecific immunity to streptococcal infection requires further study

Immunity in rheumatic fever is no more than a special case of streptococcal immunity The same antibodies are formed as in streptococcal infections which do not give rise to this delayed complication However there may be a quantitative difference since in a given epidemic of streptococcal disease the mean antibody response of rheumatic fever patients to a variety of streptococcal antigens is greater than in patients with uncomplicated infections In the case of glomerulonephritis the importance of type specific immunity is emphasized As pointed out earlier the low incident of recurrences of this disease in contrast with rheumatic fever probably depends on the fact that a single type of group A streptococcus is responsible for most of the disease Thus the development of M antibody to this type during the first attack greatly reduces the chance of a recurrence

DIAGNOSIS

Few of the many manifestations of streptococcal disease are sufficiently unique to allow for conclusive diagnosis on clinical grounds alone Scarlet fever and erysipelas exemplify the more readily recognizable streptococcal infections and classic attacks can be diagnosed with reasonable accuracy from the signs and symptoms However the findings in streptococcal pharyngitis and tonsillitis are much more variable than they were once thought to be In many cases exudate is absent and the inflammation of the pharynx cannot be distinguished from that caused by a variety of other upper respiratory infections Even when exudate or follicular tonsillitis is present the diagnosis is not certain because of the occurrence of nonstreptococcal infections which give a similar picture It must be concluded that isolation of the organism by bacteriologic culture is of primary importance in accurate diagnosis

The material for culture is most commonly obtained from the throat or the nasopharynx by the use of a sterile swab It is important to transfer the material promptly to the culture medium without allowing the swab to become dry This is done most conveniently by rolling the swab on the surface of a blood agar plate after which the inoculum is streaked out with a sterile bacteriologic loop In this way an adequate distribution of the material is usually obtained so that isolated colonies appear The nature of the blood used is of importance and sheep blood is recommended because of its property of suppressing the growth of certain hemolytic members of the genus Hemophilus However the zones of hemolysis are not always typical and some workers use both rabbit and sheep blood plates in the initial isolation of streptococci The use of blood of other species (human horse) may lead to difficulties in the recognition of hemolytic colonies and requires special care and experience In order to obtain typical colony formation it is preferable to use fresh

IMMUNITY

As noted earlier, specific antibodies to most of the extracellular products of group A streptococci are demonstrable in the serum of a large percentage of patients after recovery from streptococcal infections Similarly, certain of the surface antigens and cellular components have been shown to induce antibody response (see review in McCarty, 1954) Furthermore even though the number of individual streptococcal antigens which have been studied is relatively large, it is evident that this represents only a portion of the potential antigens These findings emphasize perhaps more clearly than in the case of any other bacterial infection, that the patient with a streptococcal infection is exposed to a wide variety of different antigenic substances during the active disease However there are few instances in which the antibodies produced can be related to immunity to infection Among the several extracellular substances for example only the antibody to the erythrogenic toxin has been shown to have a readily demonstrable effect on a disease manifestation Since the antibodies to other toxins and enzymes usually inhibit their action it is conceivable that they may affect the development of the disease process in a manner that remains undefined

In general immunity to streptococcal infection is type specific and depends on the production of antibodies to M protein This has been demonstrated both in experimental infections in laboratory animals and in natural infections in man Studies of repeated attacks of streptococcal pharyngitis in a single individual have shown that each episode is associated with a different type of group A streptococcus and that recurrent infections with the same type are extremely rare In this connection it should be noted that effective penicillin therapy can suppress the antibody response to various streptococcal antigens including the extracellular products and M protein and consequently may result in an increased incidence of second infections with a single type Following untreated infection the great majority of patients develop M antibody which is measurable by the bactericidal technic previously described There is evidence that type specific antibody may persist in detachable amounts for many years and thus provide a basis for prolonged immunity to a given type

Although type specific immunity has been established as a major factor in streptococcal disease, certain aspects of the natural history of these infections suggest the possibility of some form of nonspecific resistance or immunity Experiments with laboratory animals have contributed little in this regard In the naturally occurring disease, streptococcal infections are most common among school children and there is a progressive decrease in incidence with advancing age Considering the large number of types of group A streptococci which may be prevalent at different times it is difficult to ascribe the relative immunity of adults entirely to type specific immunity However many factors other than humoral immunity may play a role in this problem and under appropriate conditions of increased exposure as in military establishments the incidence of streptococcal infections in adults can be very high

In addition to a decrease in incidence of streptococcal infections with age there are changes in the characteristic manifestations of the disease that may be related to the immune response For example in infants under 3 years of age the disease is commonly a low grade subacute process which can last several weeks and often does not show the clear cut localizing signs that characterize streptococcal pharyngitis in the older child and adult These changes have been emphasized by Powers and Boisvert (1944) who suggested the term *streptococcosis* for all streptococcal disease in analogy with the course of events in tuberculosis The development of immunity and hypersensitivity is considered to play a major part in the changing patterns of both diseases There is evidence that the vigorous type of antibody response to a variety of streptococcal antigens in children and adults may be dependent on prior conditioning during streptococcal infections in infancy Thus it has been found (Rantz et al 1951) that the production of antistreptolysin O is often very feeble in young infants and that even in children from 1 to 6 years of age increased antibody levels are maintained for a shorter period of time than in older children In addition, the ap

tion of the organisms appears to reduce the antigenic stimulus

Hemolytic streptococci do not appear able to give rise to antibiotic resistant mutants with the ease displayed by staphylococci and certain other organisms Despite the wide spread use of penicillin in the general population there has been no significant change in the penicillin sensitivity of organisms isolated from streptococcal infections Even in the laboratory it is difficult to isolate variants of group A streptococci with significant resistance to penicillin Sulfonamide resistant mutants of a few serologic types of group A streptococci became prevalent following a mass prophylaxis experiment with sulfonamides in military populations during World War II but subsequently the incidence of these strains has greatly decreased In general it would appear that drug resistance is not a major problem in the treatment of streptococcal disease A more important complication arises from hypersensitivity of the host to the therapeutic agent Reactions of various kinds have been encountered with the sulfonamides and at the present time there is an increasing incidence of hypersensitivity to penicillin Since penicillin sensitivity may be expressed by an acute anaphylactoid reaction the results of administration of the drug to a sensitive individual can be serious Consequently care must be exercised in the use of penicillin particularly when it is given by parenteral injection

It has been established that the prompt use of penicillin therapy in streptococcal sore throat will greatly reduce the occurrence of rheumatic fever Prevention of rheumatic fever depends on elimination of the organisms and the sulfonamides are totally ineffective for this purpose When the symptoms of rheumatic fever have already developed penicillin therapy is indicated for the destruction of any streptococci remaining in the body but it appears to have little effect on the course of the acute disease However the exquisite sensitivity of group A streptococci to antibiotics has been exploited successfully in preventing recurrence of rheumatic fever in known rheumatic subjects For this purpose either penicillin or sulfonamide is effective and the continuous use of one of these agents in doses below that required for treatment of active disease decreases the risk of acquisition of a streptococcal infection and consequently the risk of recurrence of rheumatic fever

EPIDEMIOLOGY

The incidence of streptococcal infections varies in different geographic areas They are most common in the colder climates and in the north temperate zone reach a peak incidence during the winter and the early spring The apparent rarity of streptococcal infections in tropical and subtropical regions may be due in part to the fact that the disease occurs in an atypical or subclinical form since culture surveys and antibody studies indicate that the incidence of infection is higher than had been supposed on clinical grounds Crowding plays a role in the dissemination of infection The influence of this factor is illustrated by the frequency of the disease in crowded tenements and military barracks

Transmission of streptococcal infections depends primarily on intimate contact between individuals with dissemination of the organisms from the upper respiratory tract Prior to the institution of modern methods for handling and pasteurizing milk milk borne epidemics often resulted from contamination of milk by infected employes Isolated outbreaks due to infected food are still encountered occasionally but the major problem in the spread of streptococcal disease is concerned with the transfer of organisms from the respiratory tract of one individual to another

Viable streptococci are recovered readily from a variety of sources in the immediate environment of individuals who harbor the organism Thus they are found in the dust of the room in blankets on books and other objects handled by the patient These environmental reservoirs were formerly considered to be of great importance in spread of the disease but recent detailed epidemiologic studies in a military installation throw serious doubt on their significance These studies and other aspects of the epidemiology of streptococcal infections are discussed in detail by Rammelkamp (1957) The major risk of acquiring a streptococcal infection at least under the conditions of barracks life seems to be in the proximity to a carrier who is disseminating viable organisms

After spontaneous recovery from strepto-

blood agar plates or plates that have been sealed to prevent the loss of moisture during refrigeration

After overnight incubation the plates are examined for the presence of hemolytic colonies In the majority of streptococcal infections a large number of characteristic colonies *with distinct hemolytic zones will be* apparent if a satisfactory culture technic has been employed In some instances however because of the occurrence of strains which *show* little hemolysis on initial isolation or do not produce streptolysin S, the presence of streptococci may not be obvious Experience is required to recognize colonies of these strains although certain technics may assist in identifying them Thus when streaking the culture one may make a stab deep into the agar through the heavily inoculated portion of the plate so that subsurface colonies will form *which can show hemolysis as the result of* production of streptolysin O Some workers recommend the use of anaerobic culture conditions as a means of increasing the efficiency of isolating streptococci on initial culture

In view of the primary importance of group A streptococci and the possible occurrence of streptococci of other groups in the human throat further identification of the organism should be carried out by serologic grouping Since grouping antisera are now available commercially this additional step is feasible in most diagnostic laboratories For this purpose isolation of individual colonies and growth in pure culture in amounts sufficient to prepare extracts containing the group specific carbohydrate are required Typing of proved group A streptococci has a more limited application and usually is carried out only in connection with special studies

Some *confusion may result in attempting* to differentiate streptococcal carriers from patients with acute streptococcal disease by means of cultural studies However group A streptococci are not often the predominant organism in the throat of carriers and whenever these organisms are present in large numbers in association with symptoms of respiratory disease the diagnosis of streptococcal infection must be made

The use *of cultures* is of relatively little value in the diagnosis of rheumatic fever and glomerulonephritis since the initiating streptococcal infection usually has subsided before the onset of these late complications However, antibody studies have proved to be of value in establishing the occurrence of a recent streptococcal infection Thus the titers of antistreptolysin O antistreptokinase and antihyaluronidase are still increasing at the time of onset of most cases of rheumatic fever and in doubtful cases this immunologic information may be of importance in supporting *the clinical diagnosis*

TREATMENT

Prior to the introduction of the sulfonamide drugs the methods available for treatment of streptococcal infections were generally unsatisfactory Hemolytic streptococci proved to be among the most susceptible of all pathogenic bacteria to the bacteriostatic action of sulfanilamide and the discovery of this drug *began a new era in the management of* streptococcal disease The effect of the new drugs was most dramatic in the highly fatal forms of infection e g meningitis and septicemia which were seldom treated successfully by older methods The number of effective therapeutic agents has been increased by the subsequent discovery of penicillin and some of the other antibiotic drugs such as the tetracyclines since here again streptococci are *among the most susceptible of all bacteria*

Penicillin in contrast with the sulfonamides is bactericidal for streptococci, and the use of this agent is the treatment of choice in most cases of severe streptococcal disease When penicillin is used in adequate dosage for a sufficient period of time it is possible to eliminate the organisms from the upper respiratory tract so that they are no longer recoverable on culture Sulfonamides on the *other hand act by retarding the multiplication* of streptococci, which allows the natural host defenses to control the infection However viable streptococci usually remain in the respiratory tract and the sulfonamides are not effective in eliminating the carrier state The difference in mode of action of the two drugs is *reflected in their effect on antibody formation* The antistreptolysin O response of patients treated with sulfonamides is not significantly different from that of the untreated patient, while the response of penicillin treated patients is markedly depressed Thus destruc

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coccal upper respiratory infection some individuals continue to harbor the infecting organism for long periods of time In these cases streptococci can be recovered repeatedly on culture of the nasopharynx or the throat Even when cultures are negative, residual organisms may remain in lymphoid tissue as indicated by the isolation of streptococci from the deep tissues of excised tonsils The potential danger of a carrier depends on a number of factors such as the number of organisms present the biologic state of the organisms and the ease with which they are disseminated into the environment In general the ability to spread infection appears to diminish with the time which the streptococci have resided in the upper respiratory tract This decrease in infectivity is referable to qualitative changes in the organisms as well as to reduction in numbers and it has been found that in many cases streptococci tend to lose their M protein during prolonged residence in the pharyngeal tissues (Rothbard and Watson 1948)

The carrier state is not necessarily dependent on previous clinical infection During epidemic periods of streptococcal disease culture surveys of school and military populations have shown that a large proportion of the population up to one third or more may carry one of the types of group A streptococci involved in the epidemic During the pre epidemic period there is often a progressive rise in the carrier rate which precedes the occurrence of significant numbers of frank streptococcal infections Epidemics can be avoided or terminated abruptly once they occur by the use of mass penicillin prophylaxis

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11

The Mycobacteria

The mycobacteria constitute a genus (*Mycobacterium*) of the family *Mycobacteriaceae* of the order *Actinomycetales* All mycobacteria which have been intimately associated with disease of man or animals exhibiting intracellular parasitism are strongly acid fast they are not easily stainable with dyes and once stained resist decolorization by strong mineral acids The pathogenic mycobacteria include the tubercle bacilli (*Mycobacterium tuberculosis*) typical (human bovine and avian) and atypical (e g yellow bacilli and *M fortuitum*) the vole bacilli (*M microti*) the Johne s bacilli (*M paratuberculosis*) *M ulcerans* *M balnei* and the leprosy bacilli (*M leprae*) There are many species of nonpathogenic mycobacteria for example the smegma bacilli (*Mycobacterium smegmatis*) found on man the timothy bacilli (*Mycobacterium phlei*) the butter bacilli (*Mycobacterium butyricum*) and many others found in the soil (for review of taxonomic relationships see Gordon and Smith 1953) The property of acid fastness is also possessed but only to a moderate extent by a number of types of pathogenic *Nocardia* and by certain bacterial spores

The pathogenic mycobacteria cause chronic diseases with lesions of the infectious granuloma type The fully developed lesions of tuberculosis leprosy and Johne s disease have in common certain histologic characteristics collections of epithelioid and giant cells are conspicuous but the type of necrosis called caseation occurs only in tuberculosis

MYCOBACTERIUM TUBERCULOSIS

History

The tubercle bacilli cause such a wide variety of lesions and clinical symptoms in man and animals that before their discovery the pathologist and the clinician were unaware that diseases such as miliary tuberculosis tuberculous caseous pneumonia tuberculosis of cervical lymph nodes (scrofula) and lupus vulgaris (tuberculosis of the skin) had a common etiology Modern knowledge of tuberculosis began with Laennec who in 1819 described some of the prominent macroscopic aspects of tuberculous lesions and recognized the essential unity of early semitransparent tubercle and of the caseous tuberculous lesions Villemin in 1865 demonstrated that material from the human tuberculous lung could produce tuberculosis in the rabbit later he transmitted tuberculosis from cattle to rabbits

The discovery of tubercle bacilli by Robert Koch in 1882 was preceded by the discovery of the leprosy bacilli by G A Hansen in 1878 These discoveries were made without knowledge of the acid fast tinctorial proper

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organic materials—animal serum egg yolk potato extract charcoal starch etc It is this second type of medium which has been used most extensively for diagnosis and for counting single cell The failure of development of small inocula in the simple synthetic media is due primarily to the presence in the latter of substances which exert an inhibitory effect on growth and exist as impurities in the reagents or on the glassware Traces of toxic lipids and of other surface active agents are probably the most common offenders The growth inhibitory effect of many toxic agents particularly of long chain fatty acids can be neutralized by the addition of adequate amounts of animal serum (Youmans 1944) or of the albumin fraction of serum (Dubos and Davis 1946)

Indeed far from being inhibitory certain fatty acids (the C14 to C18 acids) stimulate the growth of many strains of tubercle bacilli in synthetic media to which serum albumin or other fatty acid binding substances have been added These observations have led to the development of a third, better-defined type of culture media for pathogenic mycobacteria They consist of synthetic nutrient mixtures to which are added serum albumin and either certain fatty acids usually oleic or a synthetic nontoxic water soluble ester of one of these fatty acids (Dubos and Middlebrook 1947) In the oleic acid albumin medium as in the classic media most strains of tubercle bacilli characteristically grow in the form of large clumps pellicles or heaped masses due to the hydrophobic character of the bacterial surface The water soluble esters of fatty acid commercially known as the tweens are capable of wetting the surface of tubercle bacilli and thus allowing them to grow more dispersed in a liquid medium This manner of growth has found a very useful place in experimental work whenever dispersed growth of these organisms is desired

For primary isolation of tubercle bacilli from pathologic materials an oleic acid albumin medium has been shown to be as effective as the classic complex organic media containing egg yolk etc (Middlebrook et al 1954) Microscopic examination of the surface of transparent agar oleic acid albumin medium permits earlier detection of mycobacterial colonies than is possible with the classical complex media and certain other tests such as the catalase test can be directly applied on the colonies

None of the known biochemical activities of tubercle bacilli is sufficiently characteristic to be useful in defining the group All degrees of variation exist between the various types The saprophytic strains of mycobacteria grow well on most media and their growth is in general more rapid they can often be distinguished by this character alone Moreover on complex media colonies of most strains of saprophytic mycobacteria manifest more pigmentation in the dark (usually yellow or orange) than the parasitic forms and the individual bacilli are less acid fast than virulent human bovine and avian tubercle bacilli

Resistance to Physical and Chemical Agents

Tubercle bacilli are as susceptible to desiccation heat radiation and physical agents as other nonsporulating bacteria However they are usually regarded as possessing unusual resistance to antiseptics and chemotherapeutic agents The fact that many tubercle bacilli survive exposure to acids and alkalies for prolonged periods of time is made use of in the isolation of these organisms from pathologic material However it must be emphasized that many tubercle bacilli die during this treatment and that on the other hand many other bacterial species gram positive cocci in particular can survive and grow unless inhibited by more specific means

It is probable that the hydrophobic character of the cell wall accounts in part for the resistance of tubercle bacilli to certain toxic agents It is of interest that tween 80 increases their susceptibility to a number of antibacterial agent penicillin for example probably by wetting the bacterial surface (Kirby and Dubos 1947) The resistance of tubercle bacilli to toxic agents has been somewhat exaggerated Nevertheless in practice they are less affected than other species by ordinary antiseptic agents (mineral acids alkali quaternary ammonium compounds) It is possible to add to culture media used in diagnostic work concentrations of penicillin or of malachite green which do not inhibit the growth of pathogenic mycobacteria but prevent the multiplication of gram positive

ties of these microbes staining being done by immersing the preparations for 24 hours in aqueous alkaline methyline blue solutions Ehrlich in 1882 showed that the tubercle bacilli can be stained with basic dyes in the presence of aniline oil and remain stained after treatment with strong nitric acid which decolorizes other micro organisms The acid fast staining method in common use today was developed by Ziehl, in 1882 and subsequently slightly modified by Neelsen More recently it has been found possible to stain with basic fuchsin, without heating the specimen by incorporating various surfactants in the staining mixture (Aubert 1950)

In animal lesions tubercle bacilli occur as rod shaped micro organisms On artificial cultivation they vary from coccoid to long filamentous forms depending upon the type (bovine human avian, etc), the particular strain the age of the culture and environmental conditions The typical rods are straight or somewhat bent with parallel sides and rounded ends in the animal body they vary in length from 1 to 4 μ and from 0 3 to 0 6 μ in diameter No definitive capsule has been demonstrated either in susceptible hosts or culture media

Ethanol containing 3 per cent hydrochloric acid (acid alcohol) will decolorize all rod forms of other bacteria within a few minutes but pathogenic mycobacteria including the tubercle bacilli will resist this treatment for many hours Young forms are somewhat less acid fast than older forms The acid fastness of tubercle bacilli is dependent not only upon *their chemical constitution but also upon the* integrity of their cellular structure since physical trauma is sufficient to render them non acid fast and readily stainable by an aniline dye (Yegian and Vanderlinde 1947)

Many nonpathogenic mycobacteria are less acid fast than tubercle bacilli but are nevertheless difficult to decolorize with alcohol alone therefore they are more properly designated as alcohol fast

Mycobacteria cannot be classified as gram positive or gram negative by the Gram staining technic because once they have been stained by basic dyes, they cannot be decolorized by alcohol regardless of whether or not they have been treated with iodine

It has been claimed that tubercle bacilli have a life cycle including a very small viable granular form which can pass through ordinary bacteriologic filters These claims are as yet unsubstantiated

Cultivation

The mycobacteria are strictly aerobic They can use for growth a multiplicity of simple carbon compounds as source of energy and ammonia or amino acids as source of nitrogen As yet, there is no evidence that they require any of the known vitamins except biotin (Schaefer et al , 1955) as essential growth factors They need CO for initiation of growth, some strains requiring a concentration higher than available in ordinary air (Schaefer et al , 1955) The mycobacteria grow most rapidly between pH 6 0 and 8 0 with an optimum around 6 5 to 6 8 for the pathogenic types They exhibit wide variation in optimal temperature for growth but strains pathogenic for warm blooded animals with two prominent exceptions (see *Mycobacterium ulcerans* and *balnei*) multiply most rapidly between 35° C and 40° C

Growth of most mycobacteria is slow compared with that of common bacterial species most strikingly so in the case of the human, the bovine and the murine types of tubercle bacilli the fastest reported division rate in artificial media corresponds to approximately 18 hours Growth of avian and certain atypical strains of pathogenic tubercle bacilli is more rapid particularly in media containing adequate amounts of fatty acids or fatty acid esters

Three different types of media are in general use for the cultivation of tubercle bacilli Simple synthetic media containing an abundant supply of minerals some simple source of nitrogen such as ammonia glutamate or asparagine and glucose or glycerine are capable of supporting the synthesis of large amounts of bacterial protoplasm yielding upward of 25 Gm (dry weight) per liter within 3 to 5 weeks of incubation

These results can be obtained only by inoculating such media with billions of cells and providing for intensive aeration, for example by violent shaking during incubation On the other hand growth of small inocula of many strains often of single cells can be obtained in a variety of media containing complex

Characteristics of the Different Types of *M tuberculosis*

Table 34 summarizes the known degrees of pathogenicity of the 3 principal types of tubercle bacilli for various species of animals (see Cobbett 1917) Infection tests in the rabbit and the guinea pig will usually suffice to differentiate between human bovine and avian strains freshly isolated from diseased hosts The human type has high pathogenicity for the guinea pig and low pathogenicity for the rabbit The avian type has high pathogenicity for the rabbit in addition to pathogenicity for fowl usually producing in the rabbit the so-called Yersin type* of disease and has very low pathogenicity for the guinea pig A fully virulent bovine culture has high pathogenicity for both the guinea pig and the rabbit † Strains of bovine tubercle bacilli which are somewhat attenuated may have high pathogenicity for the guinea pig but low pathogenicity for the rabbit and therefore can be confused with human strains

Voles suffering from natural tuberculosis yield strains of acid fast bacilli which differ sufficiently from the other types to be referred to as the murine type (*M microti*) This type has low pathogenicity for the guinea pig and the rabbit but is pathogenic for the mouse (Wells 1946)

Mammalian strains of tubercle bacilli (human and bovine) can usually be distinguished on primary isolation by cultural characteristics On primary isolation bovine strains grow poorly on the usual solid egg media and are inhibited by concentrations of glycerine above 0 75 per cent such cultures are called dysgonic Human type strains on the other hand usually grow more luxuriantly on the solid egg media and are more resistant to glycerine which indeed favors their growth at least as far as the potential yield is concerned they are termed eugonic Dysgonic cultures produce small smooth flat or hemispherical colonies with even entire edges on egg media eugonic cultures grow in the form of rougher usually more spreading colonies with thin uneven edges on egg media However some dysgonic cultures have human type pathogenicity and generally such distinctions between bovine and human cultures are not

* The intravenous injection of large numbers of bovine or avian tubercle bacilli into the rabbit causes a rapidly fatal septicemia with the formation of tubercles of microscopic size

† R Koch maintained that there is only one type of mammalian *M tuberculosis* The credit for distinguishing the human and the bovine types is due to Theobald Smith 1898

Table 34 Pathogenicity of Types of *M tuberculosis*

Animal Species	Types of Tubercle Bacilli			
	Bovine	*Human*	*Avian*	*Atypical*
Guinea pig	++++	++++	0	0
Rabbit	++++	+	++++	0
Mouse*	++++	+++	++++	0 or ++++
Hamster	+++	++	0	0 or +++
Anthropoids and monkey	++++	++++	0	0 or ++
Goat	+++	+	+++	?
Horse	+++	+	?	?
Dog	+++	+++	?	?
Cat	+++	+++	?	?
Cattle	++++	+	+	?
Swine	++	+	++++	?
Parrot cockatoo	++++	++++	++	?
Domestic fowl	0	0	++++	?

'0' indicates that natural infection occurs most rarely although temporarily progressive lesions may be produced by the injection of relatively large numbers of living bacilli

* Certain strains of mice are very resistant others are highly susceptible to bovine and human types (Pierce et al 1947) Mice are commonly used in experimental work at the present time

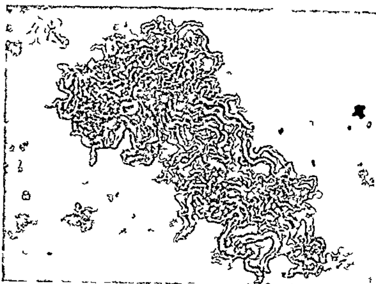


FIG 23 Photomicrograph of microcolony of strain H37Rv (fully pathogenic) showing serpentine cord formation characteristic of virulent human or bovine tubercle bacilli ×400

cocci which have resisted preliminary treatment with alkali acid or quaternary ammonium salts (Hirsch 1954)

Tubercle bacilli can survive for long periods of time in the dried state in sputa and excreta Disinfection of such materials can be effected by exposure to various anionic detergents

VARIATION AND VIRULENCE

It has long been recognized that cultures of virulent tubercle bacilli may become attenuated during prolonged repeated subcultivation on artificial media Variation in the gross morphologic appearance of cultures of mammalian tubercle bacilli has also been noted (Oatway and Steenken 1937)

However the unstable and uncontrollable physical and chemical characteristics of the commonly employed solid egg media led to much confusion in description and terminology of colony morphology

Virulent strains of human and eugonic* bovine tubercle bacilli† form microscopic 'serpentine cords' of varying thickness and length consisting of strongly acid fast bacilli oriented in parallel with the long axis of the cords (Fig 23) The formation of cords appears to be an important factor in conditioning the ability of virulent cultures to spread on the surface of liquid and solid media On the other hand those eugonic variant strains of mammalian tubercle bacilli which fail to form cords, growing in a more or less non oriented fashion are avirulent (Middlebrook et al, 1947)

Certain strains of tubercle bacilli possess relatively stable characteristics which are intermediate between those of the fully virulent cord forming strains and the completely avirulent non cord forming variants Strains of BCG (bacillus of Calmette and Guerin) fall into this group (see Immunization) Furthermore studies (Suter and Dubos 1951 Dubos et al, 1957) of the relative virulence of colonial variants of 3 different BCG strains have indicated that as was to be expected virulence is a function of other as yet unidentified properties of the bacterial cells Cord formation is a necessary but not sufficient condition for virulence of human and bovine types of tubercle bacilli

Strains of tubercle bacilli which produce cords in artificial media are able to bind the dye neutral red in the form of its red salt in alkaline aqueous media on the other hand the non cord forming variants of tubercle bacilli and nearly all saprophytic strains do not stain red under the same conditions (Dubos and Middlebrook 1948) (see Pathogenesis)

* See section on characteristics of the different types of M tuberculosis

† Nearly all such cultures on primary isolation have uniform high pathogenicity for guinea pig except cultures from cases of lupus vulgaris (tuberculosis of the skin) and from patients treated with isoniazid which often have diminished pathogenicity

chemical constituents of these organisms can vary qualitatively and quantitatively depending on the method of cultivation Nevertheless the following information is available as to the chemical constituents of mycobacteria and their relationship to the immunology and the pathology of tuberculosis (Long 1957)

The proteins synthesized by tubercle bacilli are of special significance because they elicit the tuberculin reaction It appears that all of the protein fractions of any particular type of tubercle bacilli can evoke the tuberculin reaction in an animal such as the guinea pig sensitized with the homologous type No information is available as to whether one or several specific polypeptide or protein configurations are responsible for tuberculin activity Certain protein fractions isolated from tubercle bacilli (and other mycobacteria) can induce the formation of precipitins agglutinins and complement fixing antibodies as well as anaphylactic sensitization However injection of these proteins into normal animals does not induce the delayed type of sensitivity responsible for the tuberculin reaction The proteins of human and bovine types cannot be distinguished from one another by skin sensitization The proteins of avian tubercle bacilli Johne s bacilli leprosy bacilli and saprophytic mycobacteria can be distinguished readily from those of mammalian tubercle bacilli by serologic means and by quantitative skin tests in hypersensitive individuals but there are cross reactions throughout the genus *Mycobacterium* This cross reactivity does not interfere with the practical usefulness of the tuberculin test in strongly hypersensitive individuals (see Tuberculin) Extensive studies have been made on the separation and the purification of the proteins of mycobacteria (Seibert 1950)

Tubercle bacilli contain serologically active and inactive polysaccharides of high molecular weight including glycogen (Heidelberger and Menzel 1937 Stacey 1955) The mammalian tubercle bacilli appear to contain at least two serologically distinct polysaccharides Tuberculous hosts may become hypersensitive to homologous polysaccharides this sensitization is of the anaphylactic type—distinct from the tuberculin type (Enders 1929) (see Allergy) The roles of the polysaccharides of tubercle bacilli in the immunity and the pathogenesis of tuberculosis have not been established

The mycobacteria have long been known to be very rich in lipids The systematic investigations of Anderson and his associates (Anderson 1939 1940) revealed a great variety of complex lipids branched fatty acids waxes and higher alcohols Many of the lipids are probably bound to proteins and polysaccharides in the bacterial cell some more firmly than others As the lipids are not easily dissolved or digested in the animal tissues they are probably responsible for certain aspects of the cellular reactions to tubercle bacilli (Sabin 1941) A cellular response resembling the tubercle (see Lesions Caused by Tubercle Bacilli) can be produced by crude phosphatide fractions isolated from tubercle bacilli grown in vitro Certain other lipid fractions obtained from tubercle bacilli or saprophytic mycobacteria or from unrelated source provoke similar reactions However even when these lipids of tubercle bacilli are used relatively large quantities are required to produce tuberclelike lesions or caseation necrosis There are qualitative and quantitative differences in the fatty acids of the lipids extractable from different types of mycobacteria (mammalian avian saprophytic) cultivated on the same medium and they vary with changes in the chemical composition of the medium and with the age of the cultures (Cason et al 1956 Asselineau 1956)

Recent studies of the wax fractions of mycobacteria have given more precise knowledge of the structure of the high molecular weight alpha branched chain beta hydroxy fatty acid (mycolic acids) which occur free and esterified to glycerol oligosaccharides and polysaccharides in these organisms Unesterified mycolic acids are the only lipids from tubercle bacilli which are acid fast per se An antigenic lipopolysaccharide first obtained from tubercle bacilli by Choucroun has been shown to contain in addition to mycolic acids and a serologically active polysaccharide 3 amino acids including diaminopimelic acid (Asselineau 1952)

A toxic glycolipid first extracted from living virulent tubercle bacilli by petroleum ether (Bloch 1950) has been identified as trehalose 6 6 dimycolate (Noll et al 1956) (see Pathogenesis)

reliable for conclusive type determination After repeated transfers on artificial media many dysgonic bovine cultures assume the eugonic characteristics of human cultures without change in pathogenicity or virulence

At least two types of agents of tuberculosis in human beings have been found to be non pathogenic for guinea pigs and rabbits though pathogenic for mice One type, the 'yellow bacilli causes pulmonary lesions in these animals by intravenous or airborne infection the other type *Mycobacterium fortuitum* causes renal lesions The 'yellow bacilli con stitute a well defined group of strongly acid fast bacilli larger than human or bovine bacilli They form either smooth or rough colonies on oleic acid albumin agar and are photochromogenic synthesizing a bright yel low pigment only after exposure to visible light (Buhler and Pollak 1953 Timpe and Runyon 1954) The strains classified under *Mycobacterium fortuitum* are distinguished by their lack of photochromogenicity and their comparatively rapid growth as well as their virulence for mice (Gordon and Smith 1955 Wells et al 1955 Kushner et al, 1957)

In addition to those described here other chromogenic and nonchromogenic mycobac teria easily cultivated in vitro at 37° C are undoubtedly capable of causing disease in human beings (Runyon 1955)

Avian type bacilli on primary isolation from diseased fowl or swine grow readily in the form of smooth hemispherical colonies on egg media They tend to grow somewhat more dispersed than mammalian strains in the depth of liquid media Dissociation of avian cultures to variants similar in colony morphology to those of human strains has been described These changes may or may not be associated with a change in virulence but they never are accompanied by a change in specific host pathogenicity that is such cultures never be come pathogenic for the guinea pig (Feldman, 1938)

Avian strains of tubercle bacilli are dis tinguishable from the mammalian strains by serologic methods (Furth 1926) The former possess an antigen or antigens absent in the latter and in tuberculins prepared from 'yel low bacilli,' *M fortuitum* *M ulcerans* and *M balnei* Avian strain tuberculin is also dis tinguishable from mammalian strain tuber culin by quantitative skin tests Human and bovine strain tuberculins are so similar as to be considered identical in skin tests althou h differences between protein fractions of these strains can be recognized by serologic methods (Schaefer, 1947)

Murine strains grow more slowly on egg media than do other tubercle bacilli their growth is inhibited by glycerine They are not distinguishable serologically from the human and the bovine strains (Wells, 1946)

Bacteriophages active against saprophytic and pathogenic mycobacteria have been found (Gardner and Weiser, 1947 Froman et al, 1954 Sellers et al 1957)

Pathogenic Properties of the Different Types for Man

The human and the bovine types are the principal agents of tuberculosis in man The human type is usually found in cases of pul monary tuberculosis In countries where bo vine tuberculosis is not uncommon the bovine type is often the agent of bone and joint tu berculosis and tuberculous cervical lymph adenitis which occur most often in children This apparent difference in organ specificity of the two principal mammalian types is due in reality to the fact that the route of primary infection determines in large measure the predominant organ pattern of tuberculous disease Infection by inhalation tends to pro duce pulmonary disease, whereas primary in fection by the gastro-intestinal tract (as from ingestion of unpasteurized milk) tends to re sult in the extrapulmonary patterns of disease In rural areas where cattle tuberculosis is widespread (few in the United States) the bovine type is not an uncommon agent of pulmonary tuberculosis The human type is not more pathogenic for man than the bovine type

Although there have been described a few well authenticated cases of progressive tuber culosis in man due to the avian type of tubercle bacilli (Feldman 1938 Bradbury and Young 1946) they are very rare

Chemical Constituents of Mycobacteria

The relationships between chemical con stituents and pathogenicity or virulence of mycobacteria are poorly understood The

relationship in very complex fashion and is an inseparable part of natural tuberculous infection The clinical symptoms of general toxicity in this disease are probably in large part a consequence of acquired systemic hypersensitivity to metabolic products of the parasite

In many of the typical acute infectious diseases due to bacteria the pathogenic agents multiply uninterruptedly until the host recovers or dies as in uncomplicated lobar pneumonia or diphtheria In tuberculosis however the infectious agent at first multiplies unopposed and later due to the development of some degree of acquired resistance multiplication becomes restricted but rarely are all the living parasites completely eliminated from the host Indeed a few viable tubercle bacilli may survive in the primary lesions and even after many years may originate newly progressive disease Thus there is usually established a delicate equilibrium between the host and the parasite which can shift in favor of the host or the parasite with a recurrent cycle of progressive and quiescent disease It is characteristic for tuberculosis that even in the same organ healing and progressing lesions may coexist In other words it appears that in every focus of infection a process goes on influenced in part by systemic and in part by local factors

Many studies have shown that the administration of cortisone promotes tuberculous infection in certain experimental animals In man the usual therapeutic doses of ACTH or cortisone decrease the signs and symptoms of clinical disease at least temporarily (LeMaistre et al 1951 Cocchi 1956) On the other hand these hormones can enhance the infectious process in man as well as in rabbits (Lurie 1955) Cortisone depresses but does not completely abolish hypersensitivity to tuberculin Further investigation of these phenomena may shed light on the pathogenesis and the mechanisms of resistance to tuberculosis suggesting an explanation for the adverse effects of physiologic stress on the course of the disea e and leading to new concepts of therapy

Lesions Caused by the Tubercle Bacilli

Tubercle bacilli produce in general two types of lesions One type is called exudative the other productive or proliferative The productive type is classified as an infectious granuloma, since it resembles both granulation tissue and a tumor

By examining the lungs of rabbits at different intervals of time after intravenous injection of living virulent bovine type tubercle bacilli one can observe that during the first day there is acute inflammation about the bacilli There is exudation of fluid and accumulation of polymorphonuclear leukocytes some of which contain bacilli During the succeeding few days the exudation of fluid diminishes and many of the polymorphonuclear leukocytes die monocytes appear phagocytizing both the dead leukocytes and the tubercle bacilli This exudative phase resembles pneumonia caused by pyogenic bacteria in some respects at least namely there is an exudate in the alveolar spaces and the elements of the normal tissue lung for instance are included The exudative lesion actually seen most often in the lung may be microscopic or may involve an entire lobe Its size and the rate and the violence with which it develops are functions primarily of two variables the numbers of virulent tubercle bacilli involved and the susceptibility of the host In the normal tuberculin negative host the exudation of fluid tends to be much less marked and the cellular response less vigorous In the hypersensitive (allergic) host the lesion is a very acute and labile type of re ponse seen mo t often when large numbers of bacilli reach an undiseased part of the lung by aspiration from another part with a cavity The exudative type of lesion may have a number of possible fates It may heal by resolution that is bacillary multiplication is inhibited and the bacilli may be destroyed and tissue healing may take place without car It may undergo early necrosis sometimes involving a whole lobe and lead to massive sloughing of necrotic tissue and gross ulceration with cavity formation Or it may develop into the productive type of lesion the most frequently observed lesion in tuberculosis because of its chronic character This lesion presumably develops from the exudative type in the following manner After the diminution in exudate and the appearance of mononuclear phagocytes cells of a different type epithelioid cells appear and increase in number This cell has a pale cytoplasm and a large elongated nu

The possible role of the complex lipids in the staining properties of mycobacteria has not been clearly established but it is known that the cells remain acid fast after treatment with neutral fat solvents and that treatment with 1 per cent hydrochloric acid in ether alcohol solution destroys their acid fastness This acid treatment allows extraction of certain firmly bound lipids which are otherwise not extractable from the cells with neutral fat solvents such as alcohol ether or chloroform However there is no doubt that acid fastness is not due merely to free mycolic acids alone

TUBERCULOSIS

The host parasite relationship is of such a nature in tuberculosis that the immunology and the pathology of the disease cannot be discussed separately During the course of the infection the character of the cellular response of the host changes and the parasites find modified conditions for their multiplication and dissemination The relationship between specific antigenic components of mycobacteria and protective humoral antibodies of the host has not been elucidated In tuberculosis, acquired specific resistance i e immunity, can be approached as yet only by description of events during the course of infection as seen in its morphologic pathology Therefore the pathologic aspects of tuberculosis will be emphasized here

PATHOGENESIS

Until recently there was no evidence relating any particular properties of virulent tubercle bacilli to virulence Correlation between morphologic patterns of growth and virulence led to the hypothesis that cord formation is the consequence of the accumulation of lipid material about the bacilli binding them together and surrounding them with one or more substances assumed to play a part in pathogenicity (Middlebrook 1950a) Indeed, it was shown subsequently that living or killed cells of virulent tubercle bacilli can inhibit the migration of leukocytes of normal susceptible hosts in vitro as well as in vivo (Allgower and Bloch 1949 Martin et al 1950) Virulent tubercle bacilli and certain of their culture filtrates also exert a primary toxic effect on phagocytic leukocytes resulting in leukocytic degeneration after various periods of time (Suter, 1952, Fong et al 1957) Under the same experimental conditions, non cord forming avirulent variants of these organisms and some cord forming attenuated strains appear to be much less active in these respects Finally Husseini and Elberg (1952) have observed that phthienoic, an alpha beta unsaturated, trimethylated C₂₇ fatty acid from virulent tubercle bacilli is very active in inhibiting the migration of leukocytes in vitro

Extraction of living cultures of virulent tubercle bacilli with petrol ether, a solvent which disperses virulent bacilli from their characteristic orientation yields a complex mixture of substances which inhibits leukocytic migration as do the whole bacilli (Bloch 1950) Because this fraction was extracted from the bacilli by a solvent which disrupts the characteristic cords of virulent strains, it has been called "cord factor Subsequent fractionation of the petroleum ether extracts has yielded the toxic lipid trehalose 6 6 dimycolate * The possible correlation of these findings with those of Choucroun and Asselineau and the recent isolation of a toxic lipid material from tubercle bacilli by Spitznagel and Dubos (1955) is not yet clear (Bloch et al 1957)

It seems likely that some substance or substances accounting for the characteristic serpentine "cord formation' and neutral red binding capacity of cultures of virulent tubercle bacilli play a significant part in the pathogenesis of tuberculosis but its chemical nature and the mechanism by which it may operate as a virulence factor still remains to be clarified It is significant that none of the toxic lipid fractions mentioned above binds neutral red in salt form

In addition to the subtle primary effect of virulent tubercle bacilli on the normal physiologic activity of phagocytes there develops during infection an allergic hypersensitivity to tuberculoproteins and possibly to other components of the bacteria The early appearance of hypersensitivity modifies the host parasite

* Repeated injection into mice of very small amounts of paraffin oil solution of this substance brings about hemorrhagic pulmonary lesions and death after a period of days The same fraction is much less toxic for mice when injected as a single dose even in much larger amounts

Fig 25 Bacilli in polymorphonuclear alveolitis Bacilli are more numerous than in any other tuberculous exudate Ziehl × 800 (Canetti G The Tubercle Bacillus in the Pulmonary Lesion of Man p 39 Berlin Springer)



the two types are frequently evident (Canetti 1955)

SPREAD OF TUBERCLE BACILLI IN THE HOST

Although tubercle bacilli multiply readily in artificial media in the absence of tissue cells they are primarily intracellular in the infected host However they also multiply extracellularly in ulcerated lesions such as pulmonary cavities and an extracellular phase has to be assumed in the progressive spreading infection even in nonulcerated lesions They may spread by contiguity by lymphatic drainage by the blood stream and by tubular means

Like other microscopic particulate matter

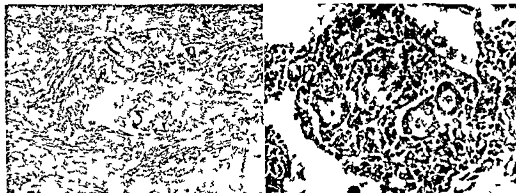


Fig 26 (*Left*) Productive lesion of lung In the center is a giant cell of enormous dimensions Assuming it to be spherical it would contain about 1 000 nuclei No bacilli by Ziehl stain Ordinary Langhans type giant cell beside it × 80

Fig 27 (*Right*) Same case Accumulation of numerous giant cells predominantly of the Langhans type No bacilli by Ziehl stain × 340 (Canetti G The Tubercle Bacillus in the Pulmonary Lesion of Man p 163 Berlin Springer)



FIG 24 Polymorphonuclear alveolitis The alveoli are filled with polymorphonuclears more or less modified Occasional macrophages and little or no fibrin This lesion can be distinguished from nontuberculous suppurative pneumonia only by the presence of bacilli × 140 (Canetti G The Tubercle Bacillus in the Pulmonary Lesion of Man p 39 Berlin Springer)

cleus The faintly outlined epithelioid cells are of irregular form and are usually arranged side by side At this time acid fast bacilli can be found in these cells but it is noteworthy that only a few cells contain stainable bacilli After 2 or 3 weeks if the lesion does not spread too rapidly a zone of proliferating fibroblasts mingled with lymphocytes appears at the periphery and in the center of the older lesions giant cells may be found These cells are characterized by their large size being up to a few hundred micra in diameter, and by their numerous dark staining nuclei situated at the periphery of the cytoplasm they also may contain stainable tubercle bacilli The typical lesion now has 3 zones a central giant cell or zone of giant cells a midzone of epithelioid cells arranged radially and a peripheral zone of fibroblasts lymphocytes monocytes and plasma cells supported by a newly formed reticulum

The cellular response just described is characteristic of the productive type of tuberculous lesion and is defined as the microscopic tubercle In human disease it is seen microscopically as a barely visible grayish translucent nodule, it may become larger, opaque and yellowish as necrosis occurs in the center of the lesion Such a macroscopic tubercle often consists of a coalesced group of microscopic tubercles Thus the tubercle may grow in size by extension or by fusion with other tubercles and become necrotic The necrotic material has the consistency and the appearance of cheese therefore, the process is called caseation necrosis The biochemical factors in the production of this peculiar type of necrosis are not clear, but it is assumed that certain autolytic enzymes of the host, which normally effect liquefaction to pus are inhibited A caseous tubercle small or large, may break into a bronchiolus or a bronchus and empty its contents, this results in cavity formation On the other hand, calcium salts may be deposited in the caseous material of the walled off tubercle The older tubercles may become surrounded by a thick layer of fibrocytes in the form of a capsule, and fibrous tissue with vascularization may penetrate and replace the tubercle Calcification and even ossification of healed pulmonary tubercles occur frequently in infancy and childhood

Of importance for the fate of the early exudative lesion are the initial number of infective tubercle bacilli their rate of multiplication in the particular host and the degree of hypersensitivity of the host A small number of infecting bacilli with little multiplication presumably leads to resolution and disappearance of the lesion, sometimes without typical tubercle formation A large initial number of tubercle bacilli, or their rapid multiplication (in a host with low resistance), especially in a highly hypersensitive host lead to early and widespread necrosis even at sites where presumably the typical tubercle has no opportunity to form (caseous and gelatinous pneumonia) Slow multiplication of tubercle bacilli, continuous or discontinuous results in the proliferative (or productive) type of lesion

The accompanying illustrations (Figs 24 to 27) show the principal characteristics of the two predominant types of tuberculous lesions It must be remembered that both the productive and the exudative types of lesions are usually present in chronic progressive tuberculosis in man they may be anatomically contiguous, and lesions intermediate between



FIG 25 Bacilli in polymorphonuclear alveolitis Bacilli are more numerous than in any other tuberculous exudate Ziehl × 800 (Canetti G The Tubercle Bacillus in the Pulmonary Lesion of Man p 59 Berlin Springer)

the two types are frequently evident (Canetti 1955)

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Like other microscopic particulate matter

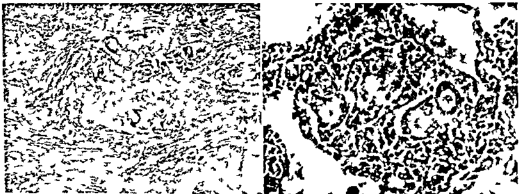


FIG 26 (*Left*) Productive lesion of lung In the center is a giant cell of enormous dimensions Assuming it to be spherical it would contain about 1 000 nuclei No bacilli by Ziehl stain Ordinary Langhans type giant cell beside it × 80

FIG 27 (*Right*) Same case Accumulation of numerous giant cells predominantly of the Langhans type No bacilli by Ziehl stain × 340 (Canetti G The Tubercle Bacillus in the Pulmonary Lesion of Man p 163 Berlin Springer)

tubercle bacilli usually reach the nearest regional lymph node by *lymphatic dissemination* There, *multiplication may take place*, and the bacterial cells may pass to other lymph nodes Thus a chain of lymph nodes may be infected and occasionally passage through the thoracic duct into the blood stream can result in systemic dissemination and miliary tuberculosis When a host is infected for the first time (regardless of whether the portal of entry is pulmonary or intestinal) gross lesions almost always develop in the draining lymph nodes For this reason the tuberculous foci of the hilar lymph nodes were called by Parrot in 1816 *miroirs des poumons* Tuberculous infection contracted by ingestion *results in the involvement of mesenteric lymph nodes* While the infection of the draining lymph nodes occurs with the regularity of biologic law during the first infection lymphatic spread is almost always absent or inconspicuous after reinfection

Hematogenous dissemination of tubercle *bacilli occurs most frequently during the progress* of first infection tuberculosis and originates as a rule either in a caseous mediastinal lymph node or by the penetration of a vein by a growing tubercle The uniform distribu*tion of tubercles in the lung as seen by roent*genograms may allow a diagnosis at this stage Rarely tubercle bacilli may be cultured from the blood Tuberculous meningitis probably a result of bacteremia occurs more frequently in children following first infection than in adults who have miliary tuberculosis

The outcome of hematogenous distribution depends on organ susceptibility The thyroid gland the pancreas the heart and the voluntary muscles almost never are the sites of miliary blood borne tubercles In parts of the lung compressed because of effusion or pneumothorax there may be fewer and smaller tubercles than elsewhere in the lung However this may be due merely to diminished blood supply

Hematogenous miliary tuberculosis may not be associated with recognizable tubercles in susceptible organs other than the lungs such as the spleen the liver or the kidneys Following hematogenous *dissemination the tubercles* in the lungs may be exudative or productive or the central part exudative surrounded by a productive zone In other organs they are as *a rule, of the productive type* Hematogenous spread may occur repeatedly

Tuberculosis of kidney, liver *spleen*, bone testes, ovaries and other organs is a consequence of blood invasion Occasionally, at necropsy of persons dying of causes other than tuberculosis, minute tuberculous lesions mostly calcified, are found in the kidneys, the liver, or the spleen

Tubular Dissemination In tubular dissemination the contents of a discharging cavity reach the bronchi with subsequent aspiration into the parenchyma of the lung initiating new pulmonary lesions Tuberculous laryngitis tonsillitis and enteritis can also result from such a spread Similarly tuberculosis of the kidney may lead to tuberculous cystitis The tubular spread of tubercle bacilli plays a conspicuous part in adult pulmonary tuberculosis For example a cavity in the upper lobe discharges its contents into a bronchus Particularly if the discharge material is liquid it may be aspirated into other portions of the same or opposite lung and there initiate new foci of disease Tuberculous caseous bronchopneumonia is apt to occur following hemoptysis *by aspiration of blood containing* viable tubercle bacilli

It can be shown in experimental animals that while the lesions of first infection continue to progress contiguously bacilli introduced into normal parts of the same organ often fail to initiate other progressive foci This experimental observation finds its clinical counterpart in the fact that progressive cavitary disease of the lungs associated with large numbers of tubercle bacilli in the bronchial secretions is not invariably followed by extensive new foci of disease Thus the resistance of individuals with active tuberculous infection is often sufficient to cope with many small local infective units of reinfection while insufficient to cope with the enormous bacillary population in the gross established lesion This fact serves as a rational basis for surgical removal of extensive areas of disease in individuals who even after surgery, continue *to shed some bacilli from smaller* lesions in other parts of the lungs

PRIMARY (FIRST INFECTION) AND POSTPRIMARY (REINFECTION) TYPES OF TUBERCULOSIS

Under the epidemiologic conditions which prevailed until recently most of the first tuberculous infections occurred in childhood This led to the use of the term childhood tuberculosis for the primary first infection type of disease and adult type for the postprimary or reinfection type of disease In reality the clinicopathologic character of tuberculosis in any individual is determined by the degree of immunity of the individual at the time of examination and not necessarily by his age Race and previous immunologic experience with the tubercle bacilli as well as many other factors are at least as important as age in determining the degree of resistance and hence the type of disease

Therefore the terms primary and postprimary are used here and defined as clinicopathologic types of disease without any necessary implications as to the age of the individual or the times of first or reinfection

An illuminating analogy of the primary and the postprimary types of tuberculosis can be recognized in experimental animals (see Immunity Koch Phenomenon) When living virulent tubercle bacilli are deposited in the skin or the lungs of a guinea pig the draining lymph nodes are soon involved and progressive disease rapidly ensues If the same guinea pig is infected subsequently in the same way again but at another site on the opposite leg or in another part of the lungs for instance the tubercle bacilli remain entirely or almost entirely localized they may multiply locally but do not or only in small numbers invade the draining lymph nodes This prevention of lymphatic spread is concomitant with the development of some degree of acquired resistance to the disease

In the United States primary tuberculosis is almost always the result of inhalation of the human type of tubercle bacilli in the form of a minute droplet nucleus less than 8 microns in diameter and containing one or very few living bacilli In countries where unpasteurized milk contaminated with bovine type tubercle bacilli is consumed the primary infection arises in the cervical lymph nodes and the intestines in an appreciable number of cases

Primary pulmonary tuberculosis differs in many respects from the postprimary type (Ghon 1916 Opie 1917) Whereas the location of the gross lesion of the primary type may be in any part of the lung and no more frequently in the apex than elsewhere the prominent postprimary lesion almost always makes its appearance near the apex A significant involvement of a hilar lymph node is present in the primary type but is not associated with the postprimary type of disease Indeed in the primary type an inconspicuous parenchymal lesion is often associated with massive caseation of the draining lymph node * whereas in the postprimary type the hilar nodes rarely contain caseous lesions even if whole lobes are diseased The primary type of tuberculosis is an acute disease healing or progressing in a relatively short time the postprimary type by contrast is more chronic because it is associated with a significant albeit inadequate degree of resistance The primary type of pulmonary tuberculosis can progress so rapidly that death occurs before cavities develop while in the postprimary type fibrosis is conspicuous and cavity formation is the rule the disease usually beginning near the apex of a lobe and progressing downward clearly through tubular routes

There are histologic differences between the progressive primary and the postprimary types of parenchymal pulmonary lesions The progressive primary type is almost always exudative the postprimary type is predominantly productive with only occasionally exudative foci usually the result of recent tubular spread The primary type often heals by resolution or may undergo caseation and calcification (without ulceration) Healing by resolution is rare in the postprimary type of disease except under the influence of effective chemotherapy

The primary type of disease most commonly seen in infants and children may be observed in adults who have escaped earlier infection On the other hand adults acquiring

* The primary pulmonary complex Ghon complex consists of the primary parenchymal lesion (Ghon tubercle") and the involved regional hilar lymphatic tissue

their first infection, as indicated by previous negative tuberculin tests, may manifest the postprimary type of disease, passing through a primary phase which remains unrecognized because of the rapid development of acquired resistance In the United States the rapidly progressive primary type is seen more often in young adult Negroes than in white adults

The postprimary "reinfection" type of tuberculosis may be caused by—seeded with—tubercle bacilli newly inhaled from without (exogenous reinfection) or by organisms which were present and had survived in the primary lesions (endogenous reinfection) The distinction between endogenous and exogenous reinfection is epidemiologically important, but it is often impossible in individual cases to ascertain the origin of the bacilli causing the postprimary disease

The healing of disease of first infection and the appearance of postprimary disease may be separated by an interval of many years The question arises as to how the relative immunity is maintained Do the tubercle bacilli in the healed primary lesion survive and thus maintain the immunologic status of the host? Are they dead but their antigens not completely destroyed and eliminated? Careful studies of healed lesions of primary type disease have shown that in most cases they contain no viable tubercle bacilli on the other hand epidemiologic data show that tuberculin sensitivity once developed is sometimes lasting The explanation in such instances may be that casual contacts with small numbers of tubercle bacilli which multiply briefly are enough to maintain tuberculin hypersensitivity and immunity without causing lesions large enough to be roentgenographically demonstrable This opinion is supported by observations on the persistence and even an increase of tuberculin hypersensitivity in American Indians long after vaccination with BCG (Aronson 1948) It may be added that the primary type of tuberculosis has been observed at autopsy in persons whose lungs had sustained a healed primary complex—healed parenchymal and hilar lymph node lesions (Terplan 1940) It is reasonable to assume that in these persons the complete healing of the first infection lesions was followed years later by complete loss of acquired resistance

IMMUNITY

The term "immunity" is used here in a restricted sense, meaning specific, acquired resistance to infection

In immunity against tuberculosis the role of *humoral* antibodies has not been convincingly demonstrated The sera of actively immunized animals agglutinate tubercle bacilli in vitro, they fix complement in the presence of tubercle bacilli and certain of their antigenic components they form precipitates with protein and carbohydrate fractions of tubercle bacilli, and they promote phagocytosis by polymorphonuclear leukocytes and monocytes in vitro Yet, such sera, even in the presence of complement have no bactericidal or lytic effects on tubercle bacilli

In many pathogenic species of bacteria the virulent variants possess surface antigens which are essential for virulence and antibodies against these confer specific resistance None of the known antigenic substances of tubercle bacilli have been shown to be related to virulence Attempts at passive transfer of acquired resistance to tuberculosis by serum transfer in experimental animals have thus far failed but the adequacy of the attempts which have been reported is subject to doubt (Raffel 1956)

Infection with tubercle bacilli enhances the host response to antigens not related to tubercle bacilli Antibody formation is nonspecifically increased and sensitization is altered (Dienes and Schoenheit, 1927) The addition of paraffin oil to killed tubercle bacilli increases and prolongs antibody formation and sensitization to the antigens of tubercle bacilli The antigenic effect of certain other antigens incorporated in paraffin oil with killed tubercle bacilli is also greatly increased and prolonged (Freund 1956) Thus it is paradoxical that protective humoral antibodies have not been shown to play a role in specific acquired resistance to tuberculosis

In addition to the formation of humoral antibodies the tuberculous host develops another type of immune process namely an altered tissue reactivity to tubercle bacilli and their components called *allergy* Certain aspects of immunity in tuberculosis are well illustrated by the reaction known as the *Koch phenomenon*

When a guinea pig is injected in the sub cutaneous tissue of the thigh with living virulent tubercle bacilli the puncture wound heals within 2 days From 10 to 14 days later a nodule appears at the site of injection It ulcerates and the ulcer usually does not heal The regional lymph nodes develop tubercles and caseate In contrast with this the already tuberculous guinea pig reacts differently to superinfection When after a period of a few weeks the infected animal is superinfected in the same way in the opposite thigh a dark colored induration develops within 2 days at the site of injection The skin over the indu rated area undergoes necrosis and soon a superficial ulcer appears However this ulcer often heals quickly Infection of regional lymph nodes is retarded or fails to develop In other words the infection has brought about two changes the host has become hypersensitive to tubercle bacilli and it has acquired the capacity to localize the superin fection Acquired resistance to infection can best be shown by infecting guinea pigs first with a strain of low virulence (BCG for ex ample) and later with a small number of viru lent bacilli The progress of the virulent super infection is much retarded but rarely entirely inhibited

Koch found that tuberculous guinea pigs react with inflammation not only to living but also to killed tubercle bacilli as well as to their protein fractions (Tuberculin) Later it was established that the Koch phenomenon can be demonstrated in guinea pigs sensitized by killed as well as by living tubercle bacilli Attempts to induce tuberculin sensitization of the delayed type with tuberculoprotein alone have been unsuccessful However it appears that the chloroform soluble wax fraction of tubercle bacilli which contains bound protein (or peptide) can confer the delayed type of tuberculin allergy on guinea pigs (Raffel 1948)

It would seem that the cells present in the tubercle play an essential role in the develop ment of allergy to protein components of tu bercle bacilli Certain aspects of this subject are dealt with in Chapter 6 particularly the difference between the tuberculin type and other types of hypersensitiveness

The Koch phenomenon is a useful paradigm of immunity experiments because it takes place in the skin and is accessible to direct observa tion Essentially the same events occur when the bacilli are introduced into the lung by inhalation or by the intravenous route

For the understanding of the mechanism of immunity in tuberculosis the fate of tubercle bacilli of reinfection and the cellular reaction to these organisms are of particular impor tance One striking difference between the fate of tubercle bacilli in the unprepared and in the immune animal is the rate of lymphatic spread as mentioned above

Krause in 1926 injected tubercle bacilli into the skin of sensitized and normal guinea pigs Early excision of the skin lesion pre vented tuberculosis in the former but not in the latter animals He felt that the retardation of lymphatic spread was due to formation of a mechanical (fibrin) barrier Does the in hibition of lymphatic dissemination depend on the prompt destruction of bacilli at their depo sition? Cultural studies of the excised lesions have shown that at the site of deposition the tubercle bacilli may multiply considerably and yet their appearance in the regional lymph nodes is retarded (Freund and Angevine 1938) Probably the specific inflammation at the site of injection promotes their local fixa tion even in the absence of gross signs of inflammation

In addition to the retardation of the lym phatic dissemination in the immune animal tubercle formation is accelerated and the mul tiplication of the newly introduced tubercl bacilli is retarded At the site of deposition of bacilli in the immune animal the accumula tion of monocytes and the appearance of epi thelioid cells is rapid the cellular reaction is nodular In the nonimmune animal the cellular response (mononuclear cells) is slow and the distribution of inflammatory cells is more dif fuse If the number of tubercle bacilli injected into the immune animal is not excessive the tubercle sometimes heals with fibrosis in the nonimmune animal it progresses and becomes caseous The number of tubercle bacilli in the lesions can be estimated by staining sections and by cultural methods As one would expect the numbers of tubercle bacilli diminish usu ally after brief preliminary multiplication in the immune animal and steadily increase in the nonimmune The tubercle bacilli at first may be seen in monocytes and later only in epithelioid cells and occasional giant cells

By counting the colonies of tubercle bacilli obtained from weighted amounts of organs of infected rabbits Lurie (1942) reinvestigated the relationship of tubercle bacilli to the cel

lular reaction to them His studies led him to conclude that the bacilli first multiply in the mononuclear phagocytes almost unopposed Later, coincident with the appearance of hypersensitiveness to tuberculin and the first stages of caseation the mononuclear cells become epithelioid cells and the numbers of bacilli diminish In the resistant host both the formation of epithelioid cells and the destruction of bacilli are rapid They are also more rapid in a more resistant organ such as the liver than in a less resistant organ such as the lung, of the same host

The tubercle its monocytes and epithelioid cells and the accelerated formation of tubercles have commonly been viewed as the means of defense of the host against the parasite, but it would seem less prejudicial to look upon them merely as anatomic evidence that changed reactivity of the host brings about conditions which limit local multiplication of the parasite

The significance of the role of allergy in immunity against tuberculosis has long been the subject of intense controversy At least 5 different viewpoints have been held on this complex problem

1 Specific acquired resistance to tuberculosis is a consequence of hypersensitivity to tuberculin and does not involve the participation of humoral antibodies against antigens of tubercle bacilli

2 Specific acquired resistance is due to humoral antibodies against one or more unidentified antigens of tubercle bacilli and is not in any way dependent upon hypersensitivity to tuberculin

3 Specific acquired resistance is due to humoral antibodies and tuberculin hypersensitivity is an undesirable accompaniment of infection tending to interfere with the protective effect of humoral antibodies

4 Specific acquired resistance is due to humoral antibodies tuberculin hypersensitivity tends to have a favorable adjuvant effect upon the operation of the specific humoral mechanism

5 Specific acquired resistance in tuberculosis operates by an as yet undemonstrated cellular mechanism unlike any hitherto known to exist in other infectious diseases and dependent upon neither humoral antibodies nor hypersensitivity to tuberculin

Let it first be made clear that many students of this problem find the available experimental evidence insufficient to warrant a decision as to which one of the above viewpoints is correct Indeed it seems probable that the many and various phases and forms of tuberculous infection and disease which occur under natural conditions or can be produced experimentally will require a more complex answer to this problem than any one of the above mentioned views indicates For example there is little doubt that the amount of caseation necrosis which can be produced by any specific number of tubercle bacilli is determined in large measure by the degree of hypersensitivity of the host and as has been pointed out above spread of bacilli by tubular routes in the lungs is dependent upon previous necrosis sloughing of the necrotic tissue and ultimate cavity formation Thus with respect to this most significant aspect of phthisis hypersensitivity is an unfavorable concomitant of tuberculous disease On the other hand the same focal necrotizing effect of hypersensitiveness probably results in decreased local multiplication of tubercle bacilli by creating an environment which is physiochemically unfavorable for their growth (Dubos 1950 Sever and Youmans 1957) particularly since tubercle bacilli are strict aerobes

It has not been possible experimentally to confer a high degree of specific acquired resistance with fractions of tubercle bacilli without the concomitance of tuberculin hypersensitivity though some increase in resistance to infection can be conferred without allergy by certain methanol extracts of tubercle bacilli (Negre 1956 Weiss and Dubos 1955) whether or not this increase in resistance is due to specific acquired immunity has not yet been established Efforts have been made to study the relationship of allergy and immunity by desensitizing immune allergic guinea pigs during the course of experimental reinfection Local and general desensitization to tuberculin can be accomplished by the repeated injection of properly spaced increasing amounts of tuberculin into hypersensitive hosts (including man) Such experiments are difficult to perform Repeated injections of large amounts of tuberculin are toxic for normal animals and dramatic focal and general deleterious effects can be produced in tuberculous hosts particularly when too large amounts of tuberculin are injected into individuals who have not yet reached the proper

degree of desensitization These investigations have been discussed at length by Rich (1951) The results of desensitization experiments strongly suggest that acquired resistance is not necessarily dependent upon cutaneous or systemic hypersensitivity to tuberculin

On the other hand passive serum transfer studies as pointed out earlier have neither proved nor excluded the possibility that the specificity of acquired resistance is mediated by a humoral mechanism

Lurie (1942) studied this problem in another way He studied the multiplication of tubercle bacilli in monocytes from normal and immune rabbits in the presence of normal and immune serum in the anterior chamber of the rabbit eye His results have been interpreted as evidence for a strictly cellular mechanism of acquired resistance independent of humoral antibodies This is probably unjustified in view of the observation that humoral antibody may appear locally at the site of transfer of washed mononuclear cells* from immunized donors to normal animals (Chase 1951) Suter (1953) and Mackaness (1954) have studied the multiplication of tubercle bacilli in monocyte cultures in vitro suppressing extracellular multiplication of the parasites by streptomycin Their contradictory results have been the subject of much controversy which is not yet resolved Fong et al (1957) have observed that monocytes from immunized guinea pigs especially when cultivated in the presence of specific immune serum are protected against the primary cytotoxic effect of virulent tubercle bacilli which otherwise can be observed within 10 hours after intracellular infection However the degree of immunologic specificity of this protective function is undefined (Elberg et al 1957) Indeed some experiments of Dubos and Schaedler (1956) indicate that some phenomena which have been interpreted as evidence of specific acquired immunity involve nonspecific tolerance mechanisms (as following injections of endotoxins)

It seems best to reserve further judgment on the subject of the specific and the nonspecific mechanism of acquired resistance to tuberculosis until further experimental data are available

In a highly infectious tuberculous environment the chance of contracting progressive clinical tuberculosis is higher in tuberculin negative persons than in those who are already tuberculin positive (Ferguson 1946) This has been observed repeatedly among student nurses and in other groups Particularly convincing are the observations made in an insane asylum where the exposure was high and considerable numbers of inmates were tuberculin negative when admitted (Flahiff 1939) Such observations in man and immunization experiments in animals have encouraged the study of prophylactic active immunization of man On the other hand where the incidence of new infections is very low as in most parts of western Europe and America today the new cases of clinically active tuberculosis occur predominantly among those older individuals in the population who are already hypersensitive to tuberculin (Palmer and Shaw 1953)

Immunization

In several species of experimental animals injection of killed tubercle bacilli moderately increases resistance to tuberculous disease This is clearly shown for example in rabbits (Opie and Freund 1937) With very small infective doses some of the immunized animals may escape the disease entirely the majority develop tuberculosis with a course slower than in the nonimmunized controls The observed degree of acquired resistance attainable with killed bacilli depends upon many as yet ill defined factors with regard to both the immunizing antigen and the mode of subsequent challenge There appears to be no strain specificity for the acquired resistance and bovine and human strains are able to cross immunize There is no evidence that the increase in resistance elicited by killed tubercle bacilli is qualitatively different from that which can be achieved by living attenuated organisms Indeed under some experimental conditions killed bacilli have been observed to be as effective as living attenuated organisms (Dubos et al 1953) Nevertheless living attenuated are more regularly effective than killed bacterial cells The question of the relative duration of acquired resistance induced by killed compared with living bacilli as well as chemical fractions thereof has not been adequately investigated

The immunizing effect of heat killed tubercle bacilli in man was studied in a hospital for

*Mononuclear cells include all leukocyte types phagocytic and nonphagocytic except polymorphonuclear leukocytes

the insane by inoculating alternate tuberculin negative patients within 2 weeks after admission (Opie et al, 1939) * The morbidity and mortality rates during the subsequent 18 months were significantly lower in the vaccinated than in the unvaccinated group

There is convincing evidence that vaccination with living strains of attenuated tubercle bacilli derived from the original attenuated strain of Calmette and Guerin (BCG) can confer some protection against naturally acquired tuberculous disease in man (Aronson 1948 Hyge 1956) The degree of protection afforded by BCG vaccination can be estimated from the report of Dahlstrom and Difs (1951) on Swedish soldiers with a moderate exposure to infection and from a recent report of the British Medical Research Council The evidence derived from this latter study indicates that BCG vaccination can confer significant protection not only against primary tuberculosis but also against the postprimary or reinfection type of disease (Hart et al, 1957)

Striking differences in invasiveness and immunizing capacity exist among the various BCG strains derived from the original culture (Jacox and Meade 1949 Suter and Dubos 1951 Dubos et al 1956) Several groups of investigators claimed in the early years of its use that BCG could dissociate' back to a virulent variant but there is no authenticated case of progressive and fatal tuberculosis in human beings attributable to such a spontaneous mutation of the vaccine On the other hand at least 4 well authenticated cases of *progressive tuberculosis in man attributable* to BCG have been reported in individuals with peculiarly inadequate ability to develop or maintain resistance to tuberculous infection (Ustvedt 1956) That BCG strains are usually less attenuated than the completely non cord forming variants of tubercle bacilli (e g H37Ra) is evident from the fact that at least 2 BCG strains have been found to cause progressive and fatal tuberculosis in silicotic guinea pigs (Vorwald et al 1950) whereas certain still more attenuated strains are unable to cause disease under these conditions

* Only tuberculin negative individuals are vaccinated with living or dead tubercle bacilli because of the Koch phenomenon which occurs in individuals who are already tuberculin positive

No basically novel technic has been introduced in practice to improve on Calmette's original method of preparing the vaccine, in spite of the fact that large variations in activity occur from one preparation to another (Fenner, 1951, Centre International de l'Enfance, 1955)

It is possible, furthermore that not only the living but also the dead bacterial cells in a BCG vaccine contribute to the development of tuberculin hypersensitivity and perhaps also to the resistance conferred by BCG vaccination

Active immunization with BCG with other attenuated strains (for example the vole bacillus) or killed bacilli has some usefulness in protecting tuberculin negative individuals However it should be recalled that persons with healed tuberculous lesions can acquire new exogenous reinfection and progressive disease Artificial immunization with the vaccines thus far offered is not likely to be more effective than previous infection with virulent tubercle bacilli Nonetheless it is desirable to have available a prophylactic vaccine such as BCG where its use is indicated (Rosenthal 1957) As of September, 1957, over 178,000 000 persons have been tuberculin tested and 66 000 000 vaccinated with BCG under the auspices of the World Health Organization

The vole bacillus is capable of inducing in guinea pigs a degree of active immunity comparable with that produced by BCG Experimental immunization of human beings with vole bacilli has been undertaken by the *British Medical Research Council* in the belief that a spontaneous change in pathogenicity of this type of tubercle bacillus for man is less likely than a spontaneous increase in virulence of the attenuated BCG strains (Wells 1946) A reasonable degree of acquired resistance to clinical tuberculosis has been observed comparable with that provided by BCG

Chemotherapy

The antimicrobial drug treatment of ulcerated tuberculosis has provided an unparalleled opportunity to study a host drug parasite relationship in clinical medicine It presents most of the problems encountered in general in chemotherapy of infectious processes and yet the response to treatment is in low mo

tion as it were in contrast with the situation in the acute infectious processes caused by more rapidly multiplying parasites

Three chemotherapeutic agents are in common clinical use at the present time for the treatment of tuberculosis streptomycin para aminosalicylic acid (PAS) and isoniazid (INH) Streptomycin exerts its therapeutic effect by bacteriostatic activity at low concentrations and bactericidal activity at higher concentration Therapeutic benefit can be achieved with this drug alone in pulmonary tuberculosis genito urinary tuberculosis miliary tuberculosis tuberculous meningo encephalitis and in the preparation of patients for thoracic surgery The degree of benefit is dependent upon many factors too complex to recount here Suffice it to state that the results attainable are conditioned by the extent of ulceration of the tuberculous lesions (e g number and size of pulmonary cavities) and the speed with which necrosis is occurring at the time of initiation of chemotherapy Thus the factors which determine the effectiveness of chemotherapy with streptomycin in tuberculosis are similar to those involved in chemotherapy of other infections in which tissue necrosis is a significant part of the disease process (McDermott 1949) The acute exudative lesion often responds dramatically while the large necrotic or partially necrotic lesions are controlled less readily and may persist to form the basis for further extensions of the disease after the cessation of chemotherapy

Tubercle bacilli like other susceptible bacteria tend to mutate to individuals resistant to streptomycin both in artificial cultures and in the tuberculous host Since in tuberculosis one is often coping with very large populations of parasite streptomycin fastness is a conspicuous problem Furthermore it has been observed that streptomycin resistant tubercle bacilli can infect other persons This factor as well as the toxicity of streptomycin for the ve tibular and auditory functions has placed marked limitations on the usefulness of this drug alone in the chemotherapy of tuberculosis

PAS exerts a bacteriostatic but little or no bactericidal effect on tubercle bacilli in vitro and has limited but recognizable chemotherapeutic activity in human disease (Therapeutic Trials Committee 1950) It is much more active against the mammalian types of tubercle bacilli than against any other microbes thus far tested Its antibacterial activity is competitively antagonized by low concentrations of para aminobenzoic acid

In 1952 the third drug of the triumvirate isonicotinic acid hydrazide (isoniazid) was discovered to have potent antimicrobial activity against tubercle bacilli in vitro, in experimental animals and in man Subsequently this compound has been found to have properties which clearly make it the most important antimicrobial chemotherapeutic agent for tuberculosis Its activity is strictly limited to tubercle bacilli it is much less active against other mycobacteria—probably useless in leprosy for example Isoniazid is active in vitro against over 99 per cent of strains of typical tubercle bacilli at levels of 0 02 to 0 2 mcg / ml and against one of the commonest types of atypical tubercle bacilli the yellow bacilli at 0 2 to 1 0 mcg /ml Claims have been made that some wild strains of typical tubercle bacilli are more resistant than this but such strains must be exceedingly rare

Isoniazid unlike streptomycin and PAS is active against intracellular tubercle bacilli Whereas streptomycin has some sterilizing activity for tubercle bacilli provided with a carbon source but no nitrogen source or no oxygen isoniazid in contrast is sterilizing for these organisms only when they are actually multiplying

In addition to streptomycin PAS and isoniazid certain other antimicrobial drugs are also used in treatment Among these pyrazinamide and cyclo erine are prominent Pyrazinamide exerts its sterilizing antibacterial effects only in acidic environments in artificial media (McDermott and Tompsett 1954) and in monocyte cultures (Mackaness 1956) The combination pyrazinamide and isoniazid exerts the greatest chemotherapeutic activity of any of the drug combinations thus far studied in experimental infections of mice (McCune et al 1956)

As with streptomycin the mutation rate of tubercle bacilli to isoniazid re istance is high enough so that in nearly 50 per cent of isoniazid treated tuberculous patients with ulcerative tuberculosis of the lungs isoniazid resistant mutants make their appearance in large numbers in the sputum if this drug is

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Three chemotherapeutic agents are in common clinical use at the present time for the treatment of tuberculosis streptomycin para aminosalicylic acid (PAS) and isoniazid (INH) Streptomycin exerts its therapeutic effect by bacteriostatic activity at low concentration and bactericidal activity at higher concentrations Therapeutic benefit can be achieved with this drug alone in pulmonary tuberculosis genito urinary tuberculosis miliary tuberculosis tuberculous meningo-encephalitis and in the preparation of patients for thoracic surgery The degree of benefit is dependent upon many factors too complex to recount here Suffice it to state that the results attainable are conditioned by the extent of ulceration of the tuberculous lesions (e g number and size of pulmonary cavities) and the speed with which necrosis is occurring at the time of initiation of chemotherapy Thus the factors which determine the effectiveness of chemotherapy with streptomycin in tuberculosis are similar to those involved in chemotherapy of other infections in which tissue necrosis is a significant part of the disease process (McDermott 1949) The acute exudative lesion often responds dramatically while the large necrotic or partially necrotic lesions are controlled less readily and may persist to form the basis for further extensions of the disease after the cessation of chemotherapy

Tubercle bacilli like other susceptible bacteria tend to mutate to individuals resistant to streptomycin both in artificial cultures and in the tuberculous host Since in tuberculosis one is often coping with very large populations of parasites streptomycin fastness is a conspicuous problem Furthermore it has been observed that streptomycin resistant tubercle bacilli can infect other persons This factor as well as the toxicity of streptomycin for the vestibular and auditory functions has placed marked limitations on the usefulness of this drug alone in the chemotherapy of tuberculosis

PAS exerts a bacteriostatic but little or no bactericidal effect on tubercle bacilli in vitro and has limited but recognizable chemotherapeutic activity in human disease (Therapeutic Trial Committee 1950) It is much more active against the mammalian types of tubercle bacilli than against any other microbes thus far tested Its antibacterial activity is competitively antagonized by low concentrations of para aminobenzoic acid

In 1952 the third drug of the triumvirate isonicotinic acid hydrazide (isoniazid) was discovered to have potent antimicrobial activity against tubercle bacilli in vitro, in experimental animals and in man Subsequently this compound has been found to have properties which clearly make it the most important antimicrobial chemotherapeutic agent for tuberculosis Its activity is strictly limited to tubercle bacilli it is much less active against other mycobacteria—probably us less in leprosy for example Isoniazid is active in vitro against over 99 per cent of strains of typical tubercle bacilli at levels of 0 02 to 0 2 mcg / ml and against one of the commonest types of atypical tubercle bacilli the yellow bacilli at 0 2 to 1 0 mcg /ml Claims have been made that some wild strains of typical tubercle bacilli are more resistant than this but such strains must be exceedingly rare

Isoniazid unlike streptomycin and PAS is active against intracellular tubercle bacilli Whereas streptomycin has some sterilizing activity for tubercle bacilli provided with a carbon source but no nitrogen source or no oxygen isoniazid in contrast is sterilizing for these organisms only when they are actually multiplying

In addition to streptomycin PAS and isoniazid certain other antimicrobial drugs are also used in treatment Among these pyrazinamide and cycloserine are prominent Pyrazinamide exerts its sterilizing antibacterial effect only in acidic environments in artificial media (McDermott and Tompsett 1954) and in monocyte cultures (Mackaness 1956) The combination pyrazinamide and isoniazid exerts the greatest chemotherapeutic activity of any of the drug combinations thus far studied in experimental infections of mice (McCune et al 1956)

As with streptomycin the mutation rate of tubercle bacilli to isoniazid resistance is high enough so that in nearly 50 per cent of isoniazid treated tuberculous patients with ulcerative tuberculosis of the lungs isoniazid resistant mutants make their appearance in large numbers in the sputum if this drug is

used alone In fact, over 90 per cent of patients who remain bacteriologically positive after 4 months of treatment with high dosage of isoniazid (8 to 16 mgm /Kg /day) excrete bacilli of which a great proportion are highly resistant to the drug (to 10 or more mcg isoniazid/ml)

Two distinct *types of isoniazid resistant mutants* have been recognized catalase *positive and catalase negative* All isoniazid susceptible human and bovine strains of tubercle bacilli have catalase activity, varying quantitatively over a fairly narrow range Catalase positive isoniazid resistant mutants can be isolated from most isoniazid susceptible populations when these are exposed to concentrations of isoniazid just above the minimal antibacterial concentration of this drug One step mutation to resistance to higher concentrations of isoniazid (1 or more mcg /ml) without complete loss of catalase is a very rare event On the other hand one step mutation to high resistance to isoniazid (10 or more mcg /ml) with complete loss of catalase is common The prevalence of such mutants is 1 in 10^4 to 1 in 10^6 in most populations of tubercle bacilli (Middlebrook 1956)

Isoniazid is metabolically altered in the human body to chemical derivatives which have little or no antimicrobial activity (Hughes 1953), and this biochemical alteration the first step of which is probably acetylation occurs at widely different rates in different individuals, though this rate remains relatively constant in any one person over long periods of time A correlation has been established between the rate of metabolic inactivation of isoniazid as determined by microbiologic assay of serum levels of the antimicrobially active drug and the types of resistant mutants—catalase positive or catalase negative—which emerge in patients with pulmonary tuberculosis under treatment with isoniazid (Mandel et al 1957) Thus serum assay for isoniazid may be used to estimate the concentrations of drug delivered to the multiplying tubercle bacilli in open tuberculous cavities and there is some evidence to suggest that success of chemotherapy with this drug depends in part on the serum level of active drug and hence upon the dose administered to the individual patient

The biochemical implications of loss of catalase activity for the mechanism of loss of isoniazid susceptibility are not clear However, it has been established that catalase deficiency almost always results in decreased pathogenicity of tubercle bacilli While very large doses of catalase negative isoniazid resistant mutants can multiply sufficiently during the first few weeks of primary infection to cause death, small inocula are unable to cause the inexorably progressive disease characteristic of virulent isoniazid susceptible strains Thus catalase negative strains can be highly *infective* in spite of their diminished pathogenicity

Catalase deficiency *is also accompanied by* increased susceptibility of the bacilli to the toxic effects of exogenous H O , although one anomalous catalase negative strain has been found to be quite resistant to H O and interestingly enough fully pathogenic Thus it has been observed that the diminished pathogenicity of isoniazid resistant strains is intimately related to their H O hypersusceptibility Therefore *it has been suggested that the concentration* of hydroperoxides in phagocytic cells increases during the development of host responsiveness in tuberculous infection (Middlebrook 1956)

Combined Drug Therapy It was early observed that the simultaneous presence of streptomycin and another unrelated antibacterial agent in culture media prevented the appearance of streptomycin resistant *mutants of* tubercle bacilli in vitro (Middlebrook and Yegian, 1946) It has also been established that clinical use of streptomycin or PAS along with isoniazid usually prevents the appearance of drug resistant tubercle bacilli in treated patients (Tuberculosis Chemotherapy Trials Committee 1955 U S Veterans Administration—Armed Forces 1957) Thus it has become common practice to use *simultaneously* at least two drugs on initiation of treatment of clinically active tuberculosis

For the best chemotherapeutic results it is necessary that the population of tubercle bacilli be characterized as to its susceptibility to the antibacterial drugs which may be used This is particularly important if there is any possibility that the patient has been treated *previously with one or more of these drugs* or has been exposed to individuals excreting drug resistant mutants

In practice if more than 1 per cent of the

population of tubercle bacilli available for drug susceptibility testing are resistant to 2 to 5 mcg streptomycin per ml of culture medium this drug is not likely to be fully effective *The analogous concentration* for isoniazid is 0 2 to 1 0 mcg and for para aminosalicylic acid 2 to 5 mcg per ml On the other hand if the population of tubercle bacilli harbored by a patient is susceptible to both streptomycin and isoniazid and these two drugs are given simultaneously and uninterruptedly in adequate dosage for a sufficient period of time there is every reason to anticipate a successful chemotherapeutic response in over 95 per cent of cases (Russell *et al* 1956) *A final evaluation of intensive combined drug approach to chemotherapy of* tuberculosis cannot be anticipated in view of the possible obstacle posed by the resting tubercle bacilli—so called 'persistors'—which escape sterilization because of their phenotypic insusceptibility to drug action Thus there is no evidence that any chemotherapeutic regimen thus far devised for tuberculosis is truly eradicative except under uncommon circumstances (see Chap 34)

Prophylactic use of chemotherapy has been suggested for (1) preventing primary infection and (2) preventing latent infection from evolving into manifest disease The administration of antimicrobial drugs for either of these separate purposes is beset by many practical problems not the least of which is motivating a healthy human being to take medications for many weeks or months Nevertheless there is evidence that the administration of isoniazid can prevent a primary infection from taking under certain experimental conditions and there is suggestive clinical evidence that antimicrobial treatment of primary infection in the highly susceptible infant is effective in preventing the early complications of the primary complex (Robinson and Meyer 1956) However the fact that isoniazid the agent which might practically be applied in prophylactic chemotherapy is unable to sterilize resting tubercle bacilli presents an obstacle the significance of which can be defined only by experience (Mitchison 1957) Co operative investigations of this problem are already under way in France and in the United States (Debre 1956 Ferebee 1956) In any case programs of control of tuberculosis by chemotherapy cannot be considered to exclude immunization because the wide variations in the exposure rate in the world today provide room for either approach *depending upon many factors* including the epidemiologic and the ecologic situation of the individual or group

Epidemiology

Within several species of animals certain experimentally inbred families are consistently more susceptible than others (Lewis and Loomis 1928 Lurie 1941) The physiologic factors manifesting these genetic differences have been studied but not yet clarified

In man the role of genetic factors in susceptibility is illustrated best in the study of tuberculosis in homozygotic and heterozygotic twins (Kallmann and Reisner 1943) If one homozygotic twin has clinical tuberculosis the other twin has 3 chances in 4 of being affected also whereas if one heterozygotic twin has the disease the other has only 1 chance in 3 of developing clinical tuberculosis Studies aimed at the demonstration of familial differences in susceptibility (Puffer 1944) are difficult *to interpret because of the complicating* variable of amount of exposure

The incidence and the type of disease are different in the American Indian the Negro and the white races the former two appearing to be more susceptible (Opie et al 1936) (Fig 28) These differences are difficult to evaluate because of the differences in living conditions It is usually assumed that the higher resistance in certain races is the result of a selection pressure occasioned by many years of *endemic infection (Ferguson 1955)* It is noteworthy in this connection that in experimental animals great differences in susceptibility within the species can be demonstrated by breeding experiments without a natural selective effect of tuberculous infection (Lurie et al 1952 Pierce et al 1947)

Curiously enough there is no striking evidence in experimental animals that age has an influence on the progress of the infection but in man it is undoubtedly true that primary tuberculosis in infants has a worse prognosis than in children or adults The death rate varies with sex and age (Fig 30) Tuberculosis in all race sex groups in the United States is becoming a disease of the older ages

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Nevertheless, for the United States as a whole, half of the newly reported active cases in 1956 were under 43 years of age The differences between men and women can be due both to basic physiologic differences associated with sex and to different degrees of exposure and living conditions Physiologic factors undoubtedly play a major role sex differences in susceptibility have also been observed in mice, especially after BCG immunization (Hoyt et al , 1957)

It is generally believed that malnutrition increases susceptibility to tuberculosis The increase in morbidity and mortality rates during wars in some countries has been attributed to concurrent malnutrition Overcrowding and other factors were also present, and their relative significance is difficult to evaluate (Dubos and Dubos 1952) It has not yet been convincingly shown that known vitamins or caloric intake have an influence on tuberculous infection in man or experimental animals However some nutritional and metabolic factors have recently been found to affect the course of tuberculous infection in mice (Dubos 1955), and in mice and hamsters at least protein intake is of great importance (Schaedler and Dubos, 1956 Ratcliffe and Merrick 1957)

The course of tuberculous infection is influenced by occupation Exposure to tubercle bacilli is frequent in the nursing and the medical professions Inhalation of dust containing silica (silicon dioxide) in certain trades such as granite cutting and certain type of mining increases the susceptibility to progressive pulmonary disease This is an important industrial health problem In experimental animals the pathologic changes induced by silica accelerate the disease in pulmonary and extrapulmonary sites wherever silica and tubercle bacilli are associated Silica has no direct effect on the parasite its local toxic action on the tissue of the host in some way promotes the disease

The distinction between infection and disease is very significant in the epidemiology of tuberculosis The infection is more widespread in urban than in rural populations The risk of infection has been declining in all parts of the United States and is now estimated to be less than 5 new infections per 1 000 young adults per year at the present time in some areas it is higher and in others it is less than 1 new infection per 1 000 young adults per annum Still in 1956 there were estimated to be at least 40 million living Americans who had been infected with tubercle

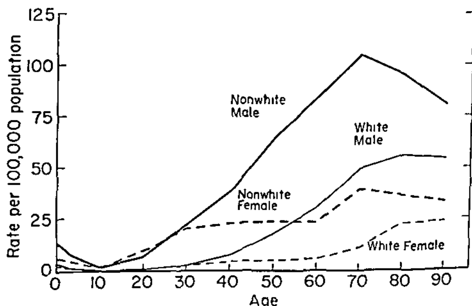


Fig 28 Age specific tuberculosis death rates by race and sex for the United States in 1956 (From 1957 Tuberculosis Chart Series U S Dept of Health Education and Welfare Division of Special Health Services)

bacilli Of these 250 000 were active cases 550 000 were currently significant inactive cases and 1 200 000 were potentially significant inactive cases (U S I H S Tuberculosis Chart Series 1957) Thus disease at any given time in the clinically progressive form is present only in a small percentage of persons infected with tubercle bacilli Chronic tuberculosis in individuals over the age of 50 is often not recognized before necropsy

Tubercle bacilli of human type are transmitted primarily by droplet infection from person to person Bacilli of the bovine type reach man usually by way of unpasteurized milk from the tuberculous cow they also can be transmitted by the same route as the human type airborne Important aspects of the infection essential for the understanding of the epidemiology of the disease are

1 Droplet infection originates from person with draining pulmonary cavities however small they are Thus pulmonary tuberculosis and cavitation specifically play major roles in the epidemiology of tuberculosis

2 Not all infections result in disease recognizable by roentgenograms or other clinical means However the degree of exposure influences to a great extent the outcome of the infection Household contact is an important factor in infection

3 The disease may have an acute flulike onset followed many months or years later by excavation of necrotic pulmonary tissue cavitation and chronic phthisis

4 In some individuals it may be fairly advanced with cavity formation and large numbers of tubercle bacilli in the sputum and produce scant if any specific clinical symptoms As carriers these individuals spread the infection

5 The progress and the type of infection are influenced by many factors such as age and previous exposure Recovery from one attack of clinical disease does not result in solid immunity

6 The reinfection type of tuberculosis commonly seen in adults is often exogenous (see Pathology)

Tuberculosis has been known in urban civilization since the beginning of recorded history In western civilization the death rate has been declining since 1870 i e before the discovery of the parasite While no longer among the leading causes of death in the United States tuberculosis is still the most frequent cause of death between the ages of 15 and 45 years and probably the principal cause of death in the world The fact that many individuals young and old are incapacitated for long periods of time by tuberculosis although they may not die of it gives it great social and economic significance The ratio of newly discovered active cases to deaths per year in the United States is higher than 10 to 1 The decrease in mortality rate from tuberculosis has not been paralleled by an equal decrease in morbidity and prevalence of infection It appears likely that the present rate of decline in mortality will continue as a result of chemotherapy but morbidity will surely decline more slowly In the Orient in Central and South America in Africa and in Asia both morbidity and mortality rates are still very high (Fig 29)

Control Measures

The spread of tuberculosis occurs in large part by long drawn out family or household epidemics in which the disease is slowly transmitted from one generation to the next (McPhedran and Opie 1935) This observation serves as a basis for most of the measures used in public health control Early diagnosis gives not only the best chance for effective treatment but also the best opportunity to prevent the spread of the disease to possible contacts It is necessary to examine persons whose risk of contracting the disease is relatively high such as *household* and *other* contacts of known cases of tuberculosis (nurses medical students house officers of hospitals) Periodic examinations of adolescent and young adults (high school and college students), applicants at large organizations governmental or private are becoming recognized routine measures For case finding the tuberculin test and x ray examination are used The value of the former consists in reducing the necessary numbers of x ray examinations in groups with a low rate of tuberculin positive persons since these do not require x ray examinations for tuberculosis *

The need for case finding work is clearly

* The percentage of reactors to tuberculin indicates the prevalence of infection in the group information of great epidemiologic interest

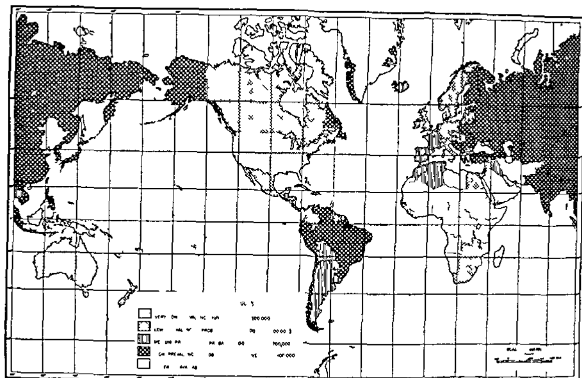


Fig 29 Prevalence of tuberculosis as estimated from the probable death rates from all forms of tuberculosis in the various countries of the world (Yelton S E 1946 Tuberculosis throughout the world Pub Health Rep *61* 1144 1160)

brought out by the fact that more than half of the cases diagnosed in ordinary medical practice are moderately or far advanced at the time of diagnosis During World War II in Massachusetts 1 4 per cent of the persons examined at induction centers for the armed forces had lesions demonstrable by chest roentgenograms (Zacks and Hyde 1944) It may be noted here that in about one half of the cases discovered by roentgenograms the infection does not tend to progress unfortunately it is not possible to predict which individuals are in this group Segregation and treatment of infectious cases (tubercle bacilli in sputum) is done best in hospitals at least for the first few months of evaluation and initiation of chemotherapy It is in the hospital that the patient can best be motivated to accept adequate chemotherapy, with or without surgery and hospitalization serves to prevent the spread of tubercle bacilli to others Unfortunately a large number of patients cannot easily be convinced that this is the wisest course to follow more than 20 per cent of the reported active cases outside of hospitals are not known to be under any kind of supervision It is estimated that in the United States there is no need for additional beds to take care of tuberculous patients, though it seems probable that better facilities are needed than are actually available Once the diagnosis of tuberculosis is made it is essential to examine periodically household and often other contacts If the patient remains at home the family has to be educated as to the mode of spread of the disease Economic problems are often conspicuous particularly when either of the parents is diseased

In the *prevention* of disease vaccination and chemotherapy of primary infections play important roles (see Immunization and Chemotherapy)

For the planning of control measures against this reportable disease accurate statistics on morbidity and mortality with reference to age sex and occupation are necessary Such information is collected by city county state Federal governmental agencies and life insurance companies Control measures are carried

out by governmental and voluntary agencies The Federal government gives financial aid to the state departments of health State, county and city departments of health are engaged in maintaining tuberculosis hospitals and clin ics with particular emphasis on case finding *among the contacts and offering diagnostic* consulting service to private practitioners The public health nurses visit families to ar range for hospitalization to obtain economic aid for the families from governmental or private welfare organizations they educate the families with reference to the care of pa tients isolation technic and personal hygiene The public health nurses are particularly help ful in the follow up of patients and contacts

The activities of voluntary private agencies are also important The largest private organ ization is the National Tuberculosis Associa tion which is supplemented by local associa tions They carry out much educational work and are active in vocational guidance and rehabilitation

The reduction of prevalence of tuberculosis caused by the bovine type during the past decades in the United States is due obviously to measures taken against tuberculosis in dairy herds and to pasteurization of milk The control measures (early diagnosis segre gation chemotherapy etc) directed against tuberculosis caused by the human type cer tainly contributed much to the phenomenal reduction in its prevalence during the past 50 years It is likely however that other fac tors had an even greater effect The frequency of this disease in different groups of people varies with their economic status It is prob able that the great rise in the standard of living (less crowding in the households better nutrition better education as to personal hy giene etc) during this century had the largest share in diminishing the prevalence of the disease It is difficult to evaluate the com parative effectiveness of the conscious public health measures and of the rise in standard of living in combating tuberculosis

Tuberculin

Old Tuberculin (O T) is prepared in the following manner Tubercle bacilli are grown on a glycerine broth or a completely synthetic medium for 4 to 6 weeks the cultures are steamed at 100 C for a few hours and then evaporated to one tenth of the original vol ume and passed through a filter to remove the bacteria The order of evaporating and filtering may be reversed It is possible to obtain fractions of the culture filtrates of tubercle bacilli possessing higher tuberculin activity per unit of dry weight than such crude preparations Tuberculin activity is as *sociated with the protein fractions of tubercle* bacilli (see Chemical Constituents of Myco bacteria) Fractions of culture filtrates of tubercle bacilli called P P D preparations (obtained either by trichloracetic acid or half saturated ammonia sulfate precipitation) are widely used No preparations of tuberculo proteins have been obtained free of polysac charide and nucleic acid Both the old tuber culin and P P D preparations have to be standardized in guinea pigs or man since bio logic activity varies with different lots *

Tuberculins made with human or bovine strains cannot be distinguished from each other but are quite different from avian tuber culin

For the Mantoux or intradermal skin test one tenth of a milliliter of a high dilution of tuberculin is injected into the skin over the forearm When active tuberculous disease is definitely suspected it is customary to use first a small dose 0 01 mg (0 1 ml of 1 10 000 dilution) and if there is no reac tion a larger dose namely 1 mg (0 1 ml of 1 100 dilution) The P P D preparation is used in amounts which produce reactions cor responding in intensity to 0 01 mg (first *strength*) 0 05 or 0 1 *mg* (*intermediate* strength) and 1 mg O T (second strength) The intermediate strength is commonly recommended for mass surveys to day It is the equivalent of 5 or 10 interna tional tuberculin units (T U) The tuberculin reaction is characterized by delayed appear ance and relatively long duration It usually appears several hours after injection and the maximum response may be seen in 1 or 2 days The reaction is considered as definitely positive if there is induration more than 10 mm in diameter erythema is usually present but is not considered in judging the size of the reaction in white persons it is commonly graded according to the longest diameter of the area indurated A papule or vesicle may develop in the central part of the indurated *area and necrosis may occur here* Weak re

* One standardized lot of P P D is commercially available for clinical u e at the present time

actions may appear and disappear faster than the stronger reactions they may be missed if the skin test is not read on the day following injection No control is necessary for the intracutaneous tuberculin test

Other forms of skin testing have been suggested to avoid the use of injections The oldest is the von Pirquet scratch test, which consists of rubbing tuberculin into a scratched area tuberculin may be incorporated into an ointment or used as a patch test (Vollmer patch) These methods of skin testing are less quantitative than the intracutaneous test

A positive tuberculin test indicates that the reactor has been infected but it does not necessarily indicate disease it is often positive in the absence of lesions recognizable by roentgenograms Lack of reaction indicates absence of infection with the following qualifications the person may be in the preallergic state during the early stage of first infection (a period not exceeding a month), or may have lost allergy due to overwhelming tuberculous infection or to unrelated infections especially measles The latter is uncommon In general persons with active lesions and those exposed recently and often (household contacts) react to smaller doses of tuberculin than others (Furcolow et al 1941) Furthermore the intensity of a positive reaction in an individual who presents no additional evidence of active disease has some prognostic significance since it has been observed that those who react more strongly are more likely to develop clinically demonstrable disease in the future The tuberculin test is of great value in excluding tuberculosis and also in epidemiologic studies to indicate the prevalence of infection Loss of tuberculin sensitivity in persons with healed tuberculosis of first infection has been observed repeatedly *in recent years* and it can be induced in experimental animals by chemotherapy Casual contacts with tubercle bacilli are becoming less frequent Thus one factor in conditioning the maintenance of tuberculin allergy is becoming less prevalent Tuberculin skin sensitivity is often lost in persons who develop Boeck s sarcoid (Reisner 1944)

False positive reactions to the larger doses of tuberculin (50 to 250 T U) occur in certain geographic areas notably across the southern part of the United States Egypt and certain parts of India (Palmer 1953) The cause or causes of these false positive reactions have not been identified yet their existence must be recognized if a true picture of the epidemiology of the infection is to be obtained

When a large amount of tuberculin, 0 5 of a milliliter of undiluted O T, for instance is injected into a tuberculous guinea pig a systemic reaction ensues quite gradually the tubercles and the surrounding tissues become congested and often hemorrhagic, exudate appears in the peritoneal and the pleural cavities and the animal may die in 24 to 72 hours What relationship this reaction bears to tuberculin allergy of the skin is not clear Anaphylactic symptoms usually fail to appear Systemic tuberculin reactions may also be produced in hypersensitive human beings and untoward effects may be produced with excessive doses in ocular tuberculosis and tuberculous cervical adenitis, but the previously disastrous consequence of such reactions can be controlled by cortisone and antimicrobial chemotherapy

LABORATORY PROCEDURES

Bacteriologic Methods

As already discussed, hypersensitivity of the skin to tuberculin indicates infection but not necessarily disease A negative test usually rules out infection A positive tuberculin reaction in an infant indicates progressive disease requiring antimicrobial chemotherapy because of their high susceptibility Conversion from negative to positive signifies recent exposure and infection frequent examinations of such cases are indicated especially because chemotherapy with isoniazid may prevent the subsequent development of clinical disease (see Chemotherapy)

Bacteriologic diagnosis rests on the demonstration of tubercle bacilli Direct smear revealing acid fast rods is usually reliable especially when present in sputum or spinal fluid In view of the fact that very large number of bacilli are necessary before they can be detected in stained film cultivation technics must be resorted to Animal inoculation may be advisable in some cases but adequate technics of cultivation almost always suffice

Direct Stained Film Morning sputum or a collection of sputa over a period of 24 hours is examined first macroscopically for possible presence of particles which should be selected for examination. In the absence of such particles a film of the sputum may be directly spread on a slide, stained and examined.

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Culture If the results of the above procedures are negative or if it is desired to prove that an acid fast rod is a tubercle bacillus (usually unnecessary), the sputum can be liquefied enzymatically or by any one of various agents (sodium hydroxide, sulfuric acid, oxalic acid, trisodium phosphate) which are bactericidal for many contaminating micro-organisms but less so for tubercle bacilli. The liquefied sputum is centrifugalized, the sediment is washed and then planted on appropriate media; the cultures should be incubated and examined at intervals for a few weeks. Nonpathogenic mycobacteria should not be confused with pathogenic strains, but atypical strains of tubercle bacilli should not be discarded as saprophytes (see Types of Tubercle Bacilli).

Animal Inoculation In some laboratories a portion of the material prepared for culture is inoculated into the groin of young guinea pigs. The animals are tested with tuberculin at intervals of a few weeks and examined for tuberculosis when the reaction becomes positive. The relative values of the cultural and the animal inoculation methods vary in different laboratories. The cultural method is to be preferred under nearly all circumstances.

The general techniques described above for sputum examination are also applicable to the examination of gastric contents (in children or when the patient cannot raise sputum), pleural fluid, urine (smegma bacilli may frequently be found in stained films of urine sediment), and spinal fluid. The fibrin web which forms after brief incubation of spinal fluid may be transferred directly to a slide and examined and cultured. Biopsy material may be frozen, then macerated and subjected to the above procedures.

Positive findings are proportional to the frequency of examinations. One or two negative findings do not rule out tuberculosis.

SEROLOGIC METHODS

Many attempts have been made to devise a serologic test to aid in the diagnosis of tuberculosis. The most widely studied serologic methods have been complement fixation reactions. Such serologic tests have given too high a percentage of false positive reactions with human sera to warrant their routine clinical use.

New types of serologic reactions have been devised recently which involve the phenomenon of adsorption of antigens of tubercle bacilli onto erythrocytes. When red cells so sensitized are exposed to sera containing antibodies against the adsorbed antigens, the red cells are agglutinated (Middlebrook and Dubos 1948, Boyden 1951). If complement is added to the mixture of sensitized red cells and specific antiserum, the red cells are lysed (Middlebrook 1950b). There is some evidence that these reactions may give a lower percentage of "false positive" reactions with human sera than the previously studied serologic tests, but the limits of diagnostic usefulness of these newer tests have not yet been defined (Gernez Rieux and Tacquet 1952).

TUBERCULOSIS IN ANIMALS

The host range of tubercle bacilli is almost unlimited. The mammalian types can infect wild animals in captivity, especially primates, and domestic animals, including dogs and cats (Hawthorne et al. 1957). Avian tubercle bacilli infect, besides fowl, swine and, rarely, cattle, sheep and horses. In aquaria fishes and turtles occasionally suffer from progressive tuberculosis caused by mycobacteria pathogenic for poikilothermic animals.

A large number of cattle are infected with the bovine type of tubercle bacilli and many have progressive disease. Tubercle bacilli are often present in the milk of infected cows even if tubercles are not demonstrable in the udder.* Most infected cattle in the United States have no gross pulmonary tuberculosis, but only infected lymph nodes; generalized tuberculosis is not frequent. The infection is diagnosed by the subcutaneous tuberculin test (local inflammatory reaction often associated with fever). The demonstration of tubercle

* In the milk of tuberculous women tubercle bacilli are very rarely found unless there are tubercles in the mammary glands, and this is very uncommon.

actions may appear and disappear faster than the stronger reactions they may be missed if the skin test is not read on the day following injection No control is necessary for the intracutaneous tuberculin test

Other forms of skin testing have been suggested to avoid the use of injections The oldest is the von Pirquet scratch test, which consists of rubbing tuberculin into a scratched area tuberculin may be incorporated into an ointment or used as a patch test (Vollmer patch) *These methods of skin testing are less* quantitative than the intracutaneous test

A positive tuberculin test indicates that the reactor has been infected but it does not necessarily indicate disease it is often positive in the absence of lesions recognizable by *roentgenograms Lack of reaction indicates ab*sence of infection with the following qualifications the person may be in the preallergic state during the early stage of first infection (a period not exceeding a month), or may have lost allergy due to overwhelming tuberculous infection or to unrelated infections, especially measles The latter is uncommon In general persons with active lesions and those exposed recently and often (household contacts) react to smaller doses of tuberculin than others (Furcolow et al 1941) Furthermore the intensity of a positive reaction in an individual who presents no additional evidence of active disease has some prognostic significance since it has been observed that those who react more strongly are more likely to develop clinically demonstrable disease in the future The tuberculin test is of great value in excluding tuberculosis and also in epidemiologic studies to indicate the prevalence of infection Loss of tuberculin sensitivity in persons with healed tuberculosis of first infection has been observed repeatedly in recent years and it can be induced in experimental animals by *chemotherapy Cas*ual contacts with tubercle bacilli are becoming less frequent Thus one factor in conditioning the maintenance of tuberculin allergy is becoming less prevalent Tuberculin skin sensitivity is often lost in persons who de*velop Boeck s sarcoid* (Reisner 1944)

False positive reactions to the larger doses of tuberculin (50 to 250 T U) occur in certain geographic areas notably across the southern part of the United States Egypt and certain parts of India (Palmer 1953) The cause or causes of these false positive reactions have not been identified yet their existence must be recognized if a true picture of the epidemiology of the infection is to be *obtained*

When a large amount of tuberculin 0 5 of a milliliter of undiluted O T , for instance is injected into a tuberculous guinea pig a systemic reaction ensues quite gradually the tubercles and the surrounding tissues become *congested* and *often hemorrhagic exudate* appears in the peritoneal and the pleural cavities and the animal may die in 24 to 72 hours What relationship this reaction bears to tuberculin allergy of the skin is not clear Anaphylactic symptoms usually fail to appear *Systemic tuberculin reactions may also* be produced in hypersensitive human beings and untoward effects may be produced with excessive doses in ocular tuberculosis and tuberculous cervical adenitis, but the previously disastrous consequence of such reactions can be *controlled* by cortisone and antimicrobial chemotherapy

LABORATORY PROCEDURES

Bacteriologic Methods

As already discussed hypersensitivity of the skin to tuberculin indicates infection but not necessarily disease A negative test usually rules out infection A positive tuberculin reaction in an infant indicates progressive disease requiring antimicrobial chemotherapy *because of their high susceptibility* Conversion from negative to positive signifies recent exposure and infection frequent examinations of such cases are indicated especially because chemotherapy with isoniazid may prevent the subsequent development of clinical disease (see Chemotherapy)

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bacilli in the milk can be but is rarely used for diagnosis For the eradication of cattle tuberculosis in the United States herds are tuberculin tested periodically and the reactors are slaughtered The rate of infection in the United States was reduced from about 4 per cent in 1917 to less than 0 5 per cent in 1950 In other countries less expensive methods are used such as slaughtering grossly tuberculous cows and segregating the reactors

Tuberculosis in the chicken is most conspicuous in the liver but the spleen the intestines the lungs and other organs are also affected Avian tubercle bacilli may be present in hens eggs The infection can be diagnosed by injection of avian tuberculin into the wattle According to a survey in 1930 in the United States about 5 8 per cent of chickens were infected with tuberculosis (more than 8 million fowl)

Pigs are infected usually with bovine or avian tubercle bacilli and rarely with the human type Infection results from ingestion of contaminated milk or material contaminated with feces of tuberculous fowl The infection in pigs is usually localized in lymph nodes of the alimentary canal

MYCOBACTERIUM ULCERANS AND MYCOBACTERIUM BALNEI

Mycobacterium ulcerans (MacCallum et al 1948) is the primary etiologic agent of a chronic or subacute type of ulceration involving both cutis and adjacent subcutaneous tissues on either the upper or the lower extremities of human beings Direct films of the exudate of such lesions reveal many acid fast rods separately in bundles or in short cords indistinguishable morphologically from mammalian tubercle bacilli Visceral lesions have not been observed The epidemiology of the disease is obscure the numbers of individuals affected are small they live in rural areas and show no suggestively significant geographic distribution Cases have been reported from Australia West Africa and Mexico

The bacilli can be isolated and cultivated on any of the usual media suitable for tubercle bacilli provided that the temperature of incubation is maintained within the limits of 25° to 35° C Their rate of multiplication approximates that of the bovine and the human types of tubercle bacilli They are pathogenic for rats mice and guinea pigs but progressive lesions occur only in those anatomic parts which normally have a temperature lower than 37° C such as the extremities the tip of the nose, the tail and the testes Thus, there is little doubt that the anatomic localization of lesions is attributable to the odd temperature requirements of this mycobacterium Of some interest from a bacteriologic as well as an epidemiologic standpoint is the fact that no other qualitative differences between this organism and the classic mammalian strains of tubercle bacilli have been observed either in serologic or in tuberculin tests

Mycobacterium balnei (Norden and Linell 1951) is another species of pathogenic mycobacteria which causes ulcerative lesions of the extremities and is unable to multiply at temperatures above 35° C Swimming pools appear to be the principal source of infection with these organisms They differ from *M ulcerans* in their much more rapid multiplication in vitro and in vivo (in the footpad of the mouse and in chick embryos) at 33 C and in some aspects of their colonial and individual morphology (Fenner 1956)

Heterologous immunity has been demonstrated in mice between *M ulcerans* and *M balnei* BCG also induces a high degree of protection against challenge infection with both species In contrast, *M ulcerans* and *M balnei* appear to be ineffective in eliciting immunity to virulent bovine tubercle bacilli (Fenner, 1957)

JOHNE S DISEASE

This is a specific enteritis of cattle sheep and deer caused by acid fast bacilli called Johne s bacilli (*M paratuberculosis*) The disease has a long incubation period runs a very chronic course characterized by intermittent diarrhea and progressive emaciation without fever In certain countries it causes serious economic loss in cattle

M paratuberculosis is a short thick rod which is acid fast and does not form spores On primary isolation it can be cultivated only on media containing an as yet unidentified substance or substances present in acid fast bacilli or in alcoholic extracts of certain plants (Twort and Ingram 1912) Recently a crystalline growth factor for *M paratuberculosis* named mycobactin has been isolated from

M phlei This substance effectively substitutes for extracts of *M phlei* as a growth factor for *M paratuberculosis* but the lag phase for growth of the latter on egg media to which mycobactin is added is still remarkably long (Rose and Snow 1955)

The lesions are characterized by gross thickening of the mucosa of the small intestine and enlargement of the mesenteric lymph nodes without ulceration The cellular reaction about the bacilli which may be intracellular or extracellular is diffuse not localized as in tuberculosis Lymphoid and epithelioid cells are present giant cells are rare caseation and calcification do not occur A skin test with johnin analogous to tuberculin is used as an aid in the diagnosis of this disease

LEPROSY

Since it is characteristically present in leprous lesions *M leprae* (Hansen s bacillus) has been accepted as the etiologic agent of leprosy although no cultures have been obtained that produce the disease in experimental animals Several strains of acid fast organisms have been cultivated from leprous lesions but those which appear to be especially significant have not been propagated in successive cultures Sera of patients with leprosy do not react specifically with the so called *M leprae* cultures

In histologic sections leprosy bacilli are from 1 5 to 8 μ long and from 0 2 to 0 5 μ in diameter They are straight or slightly curved and often occur in globular masses known as globi and in groups arranged as parallel rods As a rule leprosy bacilli are stained uniformly red with carbol fuchsin but occasionally granules can be seen Lepromin somewhat analogous to tuberculin is the name given to a suspension of lepromatous tissue rich in bacilli Mitsuda used it in skin tests as early as 1916

The disease occurs in nodular (cutaneous) and neural (anesthetic) forms Both are often present in the same patient Either of these forms may be manifested by *lepromatous tuberculoid* or rarely *intermediate types of lesions* In the lepromatous type the lesions contain many bacilli the lepromin skin test is negative and the prognosis is poor In the tuberculoid type there are few bacilli in the lesions the lepromin test is positive at 24 to 48 hours and again at 2 to 4 weeks and the prognosis is good

The tuberculin test is not affected by the presence or the absence of leprosy On the other hand patients with active tuberculosis often give a positive reaction to lepromin and BCG vaccination usually produces lepromin as well as tuberculin conversions It may be assumed from these observations that there is little or no immunologic cross reactivity between the proteins of *M tuberculosis* and *M leprae* but these two organisms possess some common antigens Indeed the sera of patients with lepromatous lesions usually contain large amounts of antibodies against one or more components of tuberculin which are adsorbable onto red cells in the hemagglutination test In nonlepromatous cases the positives are fewer and the titres lower in spite of the fact that such individuals have a better prognosis (Lowe 1955 Cochrane 1955)

In nodular leprosy the skin is raised over firm nodules (lepromata) which are most frequently present on exposed parts such as the face and the extremities The skin often ulcerates and secondary infections may set in The neural form affects peripheral particularly sensory nerves producing anesthesia Due to lack of sensation the affected hands and feet are likely to be injured leading to mutilation of these parts Lesions may develop in almost all organs except voluntary muscle The cartilage of the nose is often destroyed the mucous membranes of the mouth and the nose the eye and the testes are frequently affected Lesions are common in lymph nodes They are characterized by granulation tissue well supplied with blood vessels and lymph ducts There are many large mononuclear cells containing numerous acid fast bacilli and fat globules Multinucleated giant cells are often present Most of the acid fast bacilli are in the endothelial cells of the lymph ducts and the blood vessels In the neural lesions the bacilli are in the perineural and the epineural tissue producing extensive formation of granulation tissue The nerve fibers degenerate with loss of motor function as well as sensation Therefore the hands and the feet may undergo atrophy

The disease usually progresses very slowly the neural type is particularly insidious Re

missions often occur, *this fact has made eval*uation of possible therapeutic measures very difficult Chaulmoogra oil and its derivatives have been used for many decades in the treatment of leprosy but not until the advent of the sulfonamidelike compounds and streptomycin have unequivocal chemotherapeutic effects been demonstrable The sulfones are the drugs of choice in the treatment of certain forms of leprosy (Bushby, 1958) Isoniazid is ineffective in dosage of 3 to 5 mgm /kg /day

The bacteriologic diagnosis is most often made by finding acid fast bacilli in scrapings of the nasal mucosa and in the tissue fluid expressed after superficial incisions of the skin in certain areas There is no serologic test of value It may be noted that a large percentage of leprous patients who have neither syphilis nor yaws are Wassermann positive

The disease is believed to have originated in Central Africa whence it spread to all parts of Europe and subsequently to the rest of the world It is widely disseminated in the Orient It is estimated that there are 3 million lepers in the world, about 750 in the United States and over 30 000 in Central and South America

All human races are susceptible to the disease and it is said that susceptibility has a genetic basis This opinion is based on the greater incidence of the disease in siblings than in husbands and wives within the same family However this conclusion may not be valid susceptibility is greatest in childhood The ratio between the incidence of the disease in males and in females is approximately 2 to 1

The incubation period varies from a few months to many years most commonly from 2 to 4 years The ulcers of the skin and the nasal discharge are probably the most important sources of infection The disease is not highly contagious and probably is acquired by infecting superficial abrasions of the skin It is likely *that persons can acquire* the infection without developing manifest disease It has not been shown that animal vectors spread the infection The most effective prevention in endemic areas is the segregation of patients in leprosaria while patients in temperate climes need not be segregated The removal of children from leprous parents has proved to be a useful prophylactic measure

Unlike the tubercle bacillus *M leprae* of man does not produce progressive infection in animals But rats in many countries have a disease called "rat leprosy which is characterized by nodular skin lesions containing acid fast bacilli (*M leprae murium*) difficult to cultivate and nonpathogenic for other species of animals There is no evidence that rat leprosy can be transmitted to man or that the rat plays any role as vector for human leprosy

It is interesting to compare *M tuberculosis* and *M leprae* Tubercle bacilli have many types and their host range is almost unlimited they can be cultivated on artificial media There are only two known types of *M leprae* and they are exclusively pathogenic either for man or for rats mice and hamsters Cultivation of *M leprae* has not yet been accomplished even in tissue cultures (Hanks and Gray, 1956) Tuberculosis has been the disease of urban civilization and occurs in domestic animals Leprosy is prevalent in the tropics among people who live under rural conditions After its introduction to Europe from the Near East the disease became endemic until the 16th century and then disappeared

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12

The Staphylococci

INTRODUCTION

The staphylococci are gram positive spherical cells typically occurring in irregular clusters They grow best in the presence of oxygen and are facultatively anaerobic rare species are strictly anaerobic They usually grow well on nutrient media Their biochemical and biologic properties are variable, but they commonly ferment various carbohydrates liquefy gelatin and hemolyze blood Growth on solid media ranges in color from deep gold to white and less often, lemon yellow Staphylococci normally are parasitic on the mucous membranes and the skin, and they are responsible for many suppurative infections The strains pathogenic for man are coagulase positive

The staphylococci are members of a large group of micrococci many species of which are saprophytes and are similar morphologically to the staphylococci In the seventh edition of Bergey s *Manual of Determinative Bacteriology* (1957) the staphylococci are assigned to the genus *Staphylococcus* in the family *Micrococceae* the principal species being *Staphylococcus aureus* This represents a return to the custom of earlier classifications for in the immediately preceding edition these organisms had been placed in the genus *Micrococcus* of the same family the principal species having been designated as *Micrococcus pyogenes* var *aureus*

Infections produced by the staphylococci present a variety of clinical and pathologic forms often characterized by suppuration and ranging from mild localized pustules to fulminating rapidly fatal septicemia Furuncles and carbuncles osteomyelitis and many abscesses of soft tissues are due to the staphylococci they are frequently responsible for purulent infections of traumatic and surgical wounds, and occasionally for pneumonia meningitis or suppurative infections of the pleural the peritoneal and the synovial cavities The most common type of bacterial food poisoning is caused by staphylococci

HISTORY

Micrococci which were probably staphylococci were seen in the pus and were cultivated by several of the early bacteriologists Because of the resemblance of their clusters to a bunch of grapes Ogston in 1880 devised the name staphylococcus from the Greek word for grape (staphule) The staphylococci were isolated and studied systematically in 1884 by Rosenbach, who identified the *aureus* and the *albus* forms other varieties were studied and named in the ensuing years By the turn of the century it was recognized that staphylococci were capable of forming toxic substances, but these toxins received surprisingly little attention until interest in them was stimulated by the work of Parker and of Burnet between 1924 and 1929 Investigations of the staphylococci which followed their work resulted in the confirmation of many earlier observations and the addition of much new information about these organisms Following the studies of Dack and others beginning in 1930 staphylococcal enterotoxin was differentiated from other toxic factors and food poisoning due to the staphylococci became recognized as a clinical entity

DISTRIBUTION

The micrococci are widely distributed and are constantly present in man's environment being found in the air dust water on articles of daily use and on the skin These environmental forms are usually saprophytic The parasitic staphylococci form a part of the permanent bacterial flora of the skin and the nasopharynx On the skin they frequently lodge in hair follicles and ducts of sebaceous glands Potentially pathogenic forms are constantly carried on the skin or in the nose by approximately 20 and 50 per cent respectively of all individuals Within a week after birth a large majority of healthy infants are found to harbor staphylococci in the nose and the intestines When pathogenic staphylococci are found in the environment they may be assumed to have been derived from a human or animal source They are especially numerous in the vicinity of heavily infected individuals

Spontaneous staphylococcal infections occur in both man and animals Although man is especially susceptible to them it is not uncommon to encounter staphylococcal abscesses of the soft tissues or bone among cattle horses and other domestic animals While bovine mastitis is primarily a streptococcal disease an appreciable number of cases are caused by staphylococci

MORPHOLOGY

Typically the staphylococci are spherical cells which are grouped in irregular clusters In preparations made from broth cultures the clusters are small and single cocci pairs or short chains may be seen long chains are never found Stained preparations from agar characteristically show larger clusters of cocci In direct stained preparations of pus or other pathologic material the grouping is similar to that seen in fluid media The average size of the cocci is about 0 8 to 1 micron On the whole the staphylococci in any given culture tend to be smaller and more uniform in size and arrangement than the purely saprophytic micrococci They do not form spores and are not motile While it has generally been held that staphylococci do not possess capsules and attempts to demonstrate them usually have been unsuccessful evidence for the presence of capsular material was offered recently by Price and Kneeland (1956) A "quellung reaction was reported to be produced by immune serum which was most conspicuous with mucoid or viscid variants of pathogenic staphylococci but could also be demonstrated with nonmucoid strains The capsular material was similar in all strains encountered regardless of their serologic or bacteriophage type it could not be demonstrated in nonpathogenic strains Staphylococci stain readily with the usual basic aniline dyes less easily with some acid dyes The cocci in cultures 24 hours old or less are gram positive in older cultures some gram negative forms may be seen occasionally

CULTIVATION

The staphylococci *grow abundantly on the* usual meat extract and infusion media Both thiamine and nicotinic acid are essential *growth factors while uracil is necessary for* anaerobic growth The optimum temperature for growth is 37 C but slow development occurs at 10 C and 42 C Growth takes place at both moderately alkaline or acid reactions with an optimum at pH 7 4 The staphylococci are preferably aerobic but grow well in the absence of oxygen an atmosphere containing a mixture of air and 30 per cent carbon dioxide is particularly favorable for the production of staphylococcal toxin A few species of staphylococci are strictly anaerobic but are not commonly encountered

On agar the colonies are round raised opaque smooth and glistening usually from 1 to 2 mm in diameter and exhibit characteristic pigmentation which ranges from golden or yellow to white The pigment is developed best on agar containing a carbohydrate blood or milk The shade of pigment produced by the *aureus* varieties ranges from deep gold to pale cream While the color often is distinct after incubation for 24 hours at 37 C it is intensified when the culture is held at about 22 C for another day or two No pigment is produced anaerobically or in broth The growth in broth is uniformly turbid with an amorphous occasionally stringy sediment Distinctions based upon differences in pigmentation are responsible for the several species that were formerly recognized *Staphylococcus aureus*—golden *Staphylococcus albus*—white *Staphylococcus citreus*—lemon

yellow *Staphylococcus aurantiacus*—yellow gold and *Staphylococcus epidermis*—porcelain white When freshly isolated the majority of pathogenic staphylococci produce a golden pigment and most white strains are either nonpathogenic or only weakly pathogenic However fully pathogenic white variants of golden staphylococci are known to occur, and one cannot rely wholly on pigmentation as presumptive evidence of pathogenicity Therefore, for the purpose of this chapter, the te m *Staphylococcus aureus* will be used to signify pathogenic or potentially pathogenic staphylococci which are coagulase positive and usually are golden but may be white The significance of coagulase will be discussed in detail below

BIOCHEMICAL REACTIONS

In general gelatin is liquefied carbohydrates particularly glucose, lactose sucrose and mannitol are fermented with the production of lactic acid but no gas nitrates are reduced to nitrates milk is acidified and sometimes coagulated and indole is not formed The metabolic capacities of staphylococci vary considerably from one strain to another, a fact which reduces the significance of these reactions as a means of identification However, it should be pointed out that when freshly isolated the pathogenic staphylococci tend to exhibit a fuller complement of biochemical reactions and to show greater metabolic activity than the nonpathogenic varieties Blood agar often is hemolyzed by staphylococci this reaction being subject to the same variations as are the biochemical properties

RESISTANCE

The staphylococci are relatively more resistant to physical and chemical agents than many of the other non spore forming bacteria They remain viable for many weeks when dried in pus on cloth or other vehicles Cultures on agar are easily maintained for several months at room or refrigerator temperatures if the tubes are sealed to prevent drying of the medium The thermal death point of staphylococci is in the neighborhood of 60° C exposure to this temperature for ½ to 1 hour often being necessary to kill them The cocci resist a 1 per cent concentration of phenol for 15 minutes but are killed by a concentration of 2 per cent Certain dyes particularly crystal violet and malachite green, are strongly bactericidal for staphylococci even in low concentrations

The staphylococci are less susceptible to the bacteriostatic action of the sulfonamide drugs than the β hemolytic streptococci pneumococci or gonococci

When penicillin first was introduced as a therapeutic agent the large majority of strains of staphylococci were sensitive to it However, the succeeding years have witnessed a progressive increase in the number of penicillin resistant strains that are being isolated especially in the hospital environment Following the subsequent introduction of many of the other antibiotics a comparable change from initial sensitivity to a gradually increasing incidence of resistant strains has occurred It is generally held that the increased incidence of strains resistant to a given antibiotic is related to the extent to which that antibiotic is used therapeutically Undoubtedly, the high incidence of antibiotic resistant staphylococci must be accounted for in large part by the gradual elimination of sensitive strains through the action of antibiotics and the consequent selection and emergence of the more resistant forms Some penicillin resistant staphylococci owe their resistance to production of penicillinase It has been largely a matter of speculation whether sensitive stayphlococci could mutate to forms producing penicillinase although recently it has been reported that some penicillin sensitive strains may contain a few penicillinase producing cocci (Szybalski 1953) The chance that staphylococci may acquire resistance to penicillin in vivo appears to be slight provided that the antibiotic is administered in adequate doses in suitably selected cases but it is more likely that they can acquire resistance to certain other antibiotics during therapy Using phage typing as a means of identification strains that were originally sensitive to streptomycin or erythromycin have been reported by Rountree and Thomson and by Wise Cranny and Spink respectively to acquire resistance during treatment

ANTIGENIC RELATIONSHIPS

Both the pathogenic and the nonpathogenic staphylococci produce polysaccharides which are distinguished by differences in the end

products of their hydrolysis by their optical rotation and by specific precipitation with immune sera It is possible to separate the staphylococci by means of the precipitation reaction into 2 large groups which have been designated as Type A (pathogenic) and Type B (nonpathogenic) (Julianelle and Wieghard 1935) The polysaccharides are not themselves antigenic, the immune serum being obtained by immunization of rabbits with whole cultures of the cocci Both Types A and B staphylococci contain a protein which is antigenic but which being common to both does not serve to distinguish between the types

Thompson and Khorazo (1937) and Cowan (1938) found evidence for the existence of additional groups which were distinguished from Types A and B by precipitin reactions Cowan s additional group represents a further division of the pathogenic staphylococci while that of Thompson consists essentially of nonpathogens Since both were designated as Type C some confusion is likely to result until their position is defined more exactly Specific proteins for Types A B and Thompson s Type C were described by Verway

A rough although not entirely satisfactory distinction between pathogenic and nonpathogenic strains is obtained by simple agglutination for immune sera which agglutinate pathogenic staphylococci do not as a rule agglutinate the nonpathogenic varieties Attempts to use the technic of agglutinin absorption suggested not only a broad distinction between pathogens and nonpathogens but also the possibility of subdivisions within these groups Using agglutinin absorption and slide agglutination Cowan (1939a) demonstrated 3 distinct serologic types of pathogenic staphylococci the existence of which has been amply confirmed by others Christie and Keogh (1940) Hobbs (1948) and Oeding (1952) subsequently added several more presumably specific types which were identified by agglutination with selectively absorbed sera Some types were recognized by specific reactions with single sera while others were identified by a pattern of reactions In all instances an appreciable number of strains of *Staph aureus* could not be typed either because they were inagglutinable or because they exhibited cross reactions which prevented exact differentiation

Recently the identification of staphylococci has been attempted by bacteriophage typing in a manner similar to that used by Craigie with the typhoid bacillus The method involves the determination of similarities or differences in the susceptibility of cultures of staphylococci to a series of staphylococcal bacteriophages (Williams and Rippon 1952 Blair and Carr 1953 Jackson Dowling and Lepper 1954a) Phage typing is applicable to the study of coagulase positive staphylococci coagulase negative strains are not susceptible to the typing phages Minute drops of the phages (at predetermined "test dilutions) are deposited at spaced intervals in an established sequence on the surface of an agar plate previously seeded with the cocci to be tested Upon incubation susceptibility to the phages is shown by the development of lysis at the sites of one or more phages giving so called patterns of lysis Identical or closely related strains of staphylococci exhibit identical or closely similar patterns while differences between strains are shown by their distinctly different patterns While some variations in the patterns may be encountered the method appears to permit a more exact differentiation of individual strain of staphylococci than has been possible hitherto Four broad phage groups are recognized at present 3 of which correspond generally to Cowan s 3 serologic types Phage typing is of especial value in the study of sets of cultures that have been isolated from related sources thus it is particularly useful as a tool for the epidemiologic investigation of staphylococcal infections Among other applications the method has been employed to trace the source of staphylococci incriminated in food poisoning to identify carriers of pathogenic staphylococci and to study the relation of carriers to infections due to antibiotic resistant staphylococci The combined use of both serologic and phage typing in some epidemiologic studies has been reported

VARIATION

Variation in staphylococci sometimes occurs spontaneously and may be induced through the action of various chemical agents such as barium or lithium chloride or by exposure to antibiotics bacteriophage or irradiation Variant colonies exhibit changes in pigmentation

yellow *Staphylococcus aurantiacus*—yellow gold and *Staphylococcus epidermis*—porcelain white When freshly isolated, the majority of pathogenic staphylococci produce a golden pigment and most white strains are either nonpathogenic or only weakly pathogenic However fully pathogenic white variants of golden staphylococci are known to occur, and one cannot rely wholly on pigmentation as presumptive evidence of pathogenicity Therefore for the purpose of this chapter, the term *Staphylococcus aureus* will be used to signify pathogenic or potentially pathogenic staphylococci which are coagulase positive and usually are golden but may be white The significance of coagulase will be discussed in detail below

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ANTIGENIC RELATIONSHIPS

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proximately quantitative relationships and to be neutralized quantitatively by antitoxin The α hemolytic staphylococci produce a clear zone of hemolysis with slightly blurred edges on rabbit blood or sheep-blood agar the effect being more pronounced on the former The α hemolysin is produced by a large majority but not by all staphylococci that are pathogenic for man

The β hemolysin produces lysis of sheep and ox erythrocytes human cells are relatively resistant and rabbit cells are not affected This hemolysin is formed chiefly by staphylococci of animal origin and only occasionally by human strains Mixtures of β hemolysin and susceptible erythrocytes show no lysis during incubation for 1 hour at 37 C but lysis becomes evident when the tubes subsequently are held at room or refrigerator temperatures so-called hot-cold lysis On sheep-blood agar plates incubated at 37 C in air containing 30 per cent carbon dioxide a zone of discoloration or partial hemolysis is produced which becomes clear only after the plates are transferred to room temperatures The β hemolysin appears to be unrelated to the pathogenicity of staphylococci for man The formation of β hemolysin by a strain of *Staph aureus* would appear to be highly suggestive that it originated from an animal source

Two antigenically distinct hemolytic factors in α hemolytic culture filtrates were described by Morgan and Graydon and were designated as α_1 and α hemolysins Hemolytic culture filtrates consisted mainly of the α_1 factor α being present in the majority in varying but usually small amounts A hemolysin which was antigenically distinct from the α_1 and α lysins was described and referred to as γ hemolysin by Smith and Price (1938)

A fourth hemolytic factor called δ hemolysin was reported by Williams and Harper (1947) It is antigenically distinct from α and β hemolysins it is particularly active against rabbit and human erythrocytes and also hemolyzes the cells of several other species On blood agar δ hemolysin produces a clear zone of hemolysis with sharp margins it is more active on human and rabbit blood than on sheep blood The δ hemolysin is formed by strains producing either α or β hemolysin and appears to be related to the pathogenicity of staphylococci for man or animals a small proportion of strains produce only δ-hemolysin

While the distinction between α and β hemolysins has been clearly established some confusion exists as to the identity of the several other hemolysins Smith and Price suggested that α-hemolysin might be identical with the γ hemolysin Elek and Levy (1950) regarded α- γ and δ-hemolysins as identical while Marks (1951) considered that α- and δ hemolysins were the same but were different from γ hemolysin The α β and δ hemolysins are readily distinguished by their differences of action on rabbit sheep and human cells The α hemolysin lyses rabbit and sheep cells but not human cells β hemolysin lyses sheep cells but not rabbit or human and δ hemolysin lyses rabbit sheep and human erythrocytes The hemolytic activity of γ hemolysin parallels that of δ hemolysin The β hemolysin may be produced anaerobically under which condition α and δ-hemolysins are inhibited

Leukocidin is produced in varying amounts by the majority of pathogenic staphylococci and is formed independently of exotoxin Both leukocidin and exotoxin are often present in the same culture filtrates and both destroy rabbit leukocytes because of this the effect on leukocytes as determined by the Neisser Wechsberg technic which is based upon the capacity of living rabbit leukocytes to reduce methylene blue has led to the assumption that the two factors are identical That leukocidin is distinct from exotoxin is shown when Valentine s method (1936) is used which involves the direct microscopic examination of stained preparations made from a mixture of culture filtrate and human blood Since human leukocytes are affected by leukocidin but not by exotoxin any destruction of the leukocytes which is observed may be thus attributed to the action of leukocidin Leukocidin is soluble filterable more labile than exotoxin and is antigenic

Certain strains of staphylococci produce an enterotoxin which is responsible for the acute gastro intestinal symptoms of food poisoning (Dack 1956) Although enterotoxigenic strains are widely distributed they appear to comprise a relatively small proportion of all the pathogenic staphylococci By coincidence many food poisoning strains produce exotoxin

and may appear as rough mucoid or as minute G forms Alterations in metabolic or pathogenic properties may take place Antigenic differences between variants and the parent strain sometimes can be demonstrated as well as differences in chemical composition Avirulent small colony G forms were produced by Wise and Spink (1954) with the aid of penicillin erythromycin carbomycin and bacitracin They could be maintained in media containing sublethal concentrations of antibiotic but reverted to the virulent large colony form when transferred to antibiotic free media There have been several reports of the primary isolation of G forms from human pathologic sources

TOXINS AND ENZYMES

The staphylococci elaborate several toxins and enzymes which are presumed to contribute in varying degrees to their ability to produce infection

A soluble filterable thermolabile exotoxin is produced which causes tissue necrosis or death in experimental animals and hemolyzes rabbit erythrocytes It is produced chiefly by staphylococci which are pathogenic for man From 0 0005 to 0 002 ml of a potent exotoxin hemolyzes 1 per cent rabbit cells dermonecrosis is produced by 0 001 to 0 005 ml when injected intradermally, and in an intravenous dose of 0 25 to 0 5 ml per kilo of body weight the toxin is rapidly lethal for rabbits Exotoxin *is antigenic giving rise to a specific antitoxin which neutralizes it according to the law of multiple proportions the potency of antitoxin* is measured in terms of a standard international unit An antigenic toxoid may be prepared by treating the exotoxin with formalin

Four hemolysins have been described which are designated as α β, γ and δ hemolysin respectively (Glenny and Stevens 1938 Smith and Price 1938 Williams and Harper 1947) They are distinguished by certain differences in their properties and each is antigenically distinct from the others (Table 35) Rabbit erythrocytes are particularly susceptible to lysis by α hemolysin this is less active on sheep cells and its action on human erythrocytes is negligible There is a difference of opinion as to whether α hemolysin is identical with the lethal and necrotizing factors however the 3 factors often have been reported to be present in toxic culture filtrates in ap

TABLE 35 SOME PROPERTIES OF STAPHYLOCOCCAL HEMOLYSINS

	α HEMOLYSIN	β HEMOLYSIN	γ HEMOLYSIN	δ HEMOLYSIN
Species of susceptible erythrocytes	Rabbit Sheep	Sheep Ox	Rapid lysis Rabbit Human *Delayed lysis* sheep guinea pig ox rat horse	Human rabbit horse sheep rat guinea pig
Produced by	Strains chiefly of human origin	Strains chiefly of animal origin	Strains producing α hemolysin	Strains producing α hemolysin
Optimum temperature (hemolysis of cell suspension)	37° C	37° C (1 hr) followed by 4° C or 22° C overnight	37° C	37° C
Inactivation by heat	55 65° C (30 min) (Not completely destroyed in 30 min at 100° C)	More resistant than α hemolysin at 57° C (30 min)	55° C (30 min)	Partial inactivation at 65° C (30 min)
Toxicity for experimental animals	Strongly dermonecrotic for rabbit Rapidly lethal in small doses for rabbit and mouse	Transient erythema rabbit and guinea pig Lethal for rabbit in large doses not for guinea pig or mouse	Slightly dermonecrotic for rabbit and guinea pig Lethal in 10 to 45 hr for rabbit not for guinea pig or mouse	Edema and induration no necrosis in rabbit and guinea pig Lethal effect unknown

proximately quantitative relationships and to be neutralized quantitatively by antitoxin The α hemolytic staphylococci produce a clear zone of hemolysis with slightly blurred edges on rabbit blood or sheep blood agar, the effect being more pronounced on the former The α hemolysin is produced by a large majority but not by all staphylococci that are pathogenic for man

The β hemolysin produces lysis of sheep and ox erythrocytes human cells are relatively resistant and rabbit cells are not affected This hemolysin is formed chiefly by staphylococci of animal origin and only occasionally by human strains Mixtures of β hemolysin and susceptible erythrocytes show no lysis during incubation for 1 hour at 37 C but lysis becomes evident when the tubes subsequently are held at room or refrigerator temperatures so called hot cold lysis On sheep-blood agar plates incubated at 37 C in air containing 30 per cent carbon dioxide a zone of discoloration or partial hemolysis is produced which becomes clear only after the plates are transferred to room temperatures The β hemolysin appears to be unrelated to the pathogenicity of staphylococci for man The formation of β hemolysin by a strain of *Staph aureus* would appear to be highly suggestive that it originated from an animal source

Two antigenically distinct hemolytic factors in α hemolytic culture filtrates were described by Morgan and Graydon and were designated as α_1 and α hemolysins Hemolytic culture filtrates consisted mainly of the α_1 factor α being present in the majority in varying but usually small amounts A hemolysin which was antigenically distinct from the α_1 and α lysins was described and referred to as γ hemolysin by Smith and Price (1938)

A fourth hemolytic factor called δ hemolysin was reported by Williams and Harper (1947) It is antigenically distinct from α and β hemolysins it is particularly active against rabbit and human erythrocytes and also hemolyzes the cells of several other species On blood agar δ hemolysin produces a clear zone of hemolysis with sharp margins it is more active on human and rabbit blood than on sheep blood The δ hemolysin is formed by strains producing either α or β hemolysin and appears to be related to the pathogenicity of staphylococci for man or animals a small proportion of strains produce only δ hemolysin

While the distinction between α and β hemolysins has been clearly established some confusion exists as to the identity of the several other hemolysins Smith and Price suggested that α hemolysin might be identical with the γ hemolysin Flek and Levy (1950) regarded α γ and δ hemolysins as identical while Marks (1951) considered that α and δ hemolysins were the same but were different from γ hemolysin The α β and δ hemolysins are readily distinguished by their differences of action on rabbit sheep and human cells The α hemolysin lyses rabbit and sheep cells but not human cells β hemolysin lyses sheep cells but not rabbit or human and δ hemolysin lyses rabbit sheep and human erythrocytes The hemolytic activity of γ hemolysin parallels that of δ hemolysin The β hemolysin may be produced anaerobically under which condition α and δ hemolysins are inhibited

Leukocidin is produced in varying amounts by the majority of pathogenic staphylococci and is formed independently of exotoxin Both leukocidin and exotoxin are often pre ent in the same culture filtrates and both destroy rabbit leukocytes because of this the effect on leukocytes as determined by the Neisser Wechsberg technic which is based upon the capacity of living rabbit leukocytes to reduce methylene blue has led to the assumption that the two factors are identical That leukocidin is distinct from exotoxin is shown when Valentine s method (1936) is used which involves the direct microscopic examination of stained preparations made from a mixture of culture filtrate and human blood Since human leukocytes are affected by leukocidin but not by exotoxin any destruction of the leukocytes which is observed may be thus attributed to the action of leukocidin Leukocidin is soluble filterable more labile than exotoxin and is antigenic

Certain strains of staphylococci produce an enterotoxin which is responsible for the acute gastro intestinal symptoms of food poisoning (Dack 1956) Although enterotoxigenic strains are widely distributed they appear to comprise a relatively small proportion of all the pathogenic staphylococci By coincidence many food poisoning strains produce exotoxin

and may appear as rough, mucoid or as minute G forms Alterations in metabolic or pathogenic properties may take place Antigenic differences between variants and the parent strain sometimes can be demonstrated as well as differences in chemical composition Avirulent small colony G forms were produced by Wise and Spink (1954) with the aid of penicillin erythromycin carbomycin and bacitracin They could be maintained in media containing sublethal concentrations of antibiotic but reverted to the virulent large colony form when transferred to antibiotic free media There have been several reports of the primary isolation of G forms from human pathologic sources

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lished in the tissues or to produce disease. In fact, it has not been shown conclusively, nor has it been disproved, that the products demonstrated in the laboratory contribute to the pathogenic activity of the staphylococci in the body. Nevertheless investigations on the staphylococci and their products have provided information which carries certain implications concerning the mechanisms of their pathogenesis. Some of the soluble products of the staphylococci have been studied in a relatively crude form. Their role could be evaluated better by the use of more highly purified preparations, and the knowledge so gained also could be of material assistance in studies on immunity. Fortunately, promising efforts are being made in this direction.

The pathogenic capacity of *Staph aureus* involves the ability both to become established in the body and to be maintained in the tissues. To accomplish this, the cocci must elaborate substances which either play a direct role in their aggression or serve to counteract the defenses of the host. It appears possible, for example, that strains differ in enzyme producing capacities. In certain generalized infections exotoxin appears to be responsible for the clinical manifestations of severe toxemia and possibly contributes to the death of young persons. It would seem unlikely that any important role could be attributed to α hemolysin since this hemolysin has little or no action on human erythrocytes. Staphylococcal food poisoning results directly from the ingestion of preformed enterotoxin in the food eaten. In the majority of staphylococcal infections the predominating feature is the production of a localized lesion. The local establishment of the cocci may be aided by the necrotizing action of exotoxin or the destruction of leukocytes by leukocidin, but outside of the area of local involvement the effect of toxin would appear to be negligible.

Rogers and Tompsett (1952) have shown that coagulase positive staphylococci are phagocytized *readily under conditions in which* pneumococci, group A streptococci and Friedlander's bacilli are rarely ingested. Furthermore, they have demonstrated that the pathogenic staphylococci are able to survive and multiply within the leukocytes with ultimate destruction of the latter and liberation of the cocci. *Under similar conditions* coagulase negative strains are unable to multiply or to survive after ingestion. Recognizing that such factors as exotoxin, leukocidin and coagulase may contribute to the intraleukocytic survival of pathogenic staphylococci, these authors suggest that the fact of survival within the leukocytes may enhance the ability of the cocci to produce infection in man.

The high correlation between pathogenicity and the ability of staphylococci to clot blood plasma has led to numerous suggestions as to the role of coagulase in infection. The pertinent observation was made by Smith and Hale (1944) that only those animal species are susceptible to staphylococcal infections whose plasma can be clotted by staphylococci. Hale and Smith (1945) demonstrated that phagocytosis is inhibited in vitro and suggested that *this mechanism might be operative in the* body. There is some question whether the in vitro reactions of coagulase can be reproduced in the body. Menkin and Walston were unable to produce blockage of the lymphatics by coagulase, and Fisher could not demonstrate either microscopically or grossly that intravascular clotting occurred during the course of infection. On the other hand, it has been suggested that by laying down a fibrin barrier coagulase may take part in the development of the staphylococcal abscess. In support of this view, Rammelkamp and Lebovitz (1956) have offered evidence that the concentration of coagulase reacting factor in the serum is significantly higher in adults, in whom the tendency for an infection to localize is greatest, than in infants and young children. Ekstedt (1956) has postulated that the ability of staphylococci to grow in human serum may be related to a *protective action of* coagulase against the antibacterial effect of the serum. Other substances, as Type A polysaccharide or α and β toxins, had no such neutralizing action on the serum.

While a spreading factor might aid in the dispersion of the cocci and their toxic products, *this effect would seem to be* contrary to the usual tendency of staphylococci to produce localized lesions. It has been suggested that a more likely role of hyaluronidase would be found in its ability to depolymerize hyaluronic acid in the connective tissues and thus to remove a potential inhibitor to the in vivo formation of enzymes and toxins by the cocci.

as well as enterotoxin However, the enterotoxin is a distinct entity and is distinguished from other staphylococcal toxins by certain physical properties and by its *antigenic specificity* It is generally held that enterotoxin resists boiling for 30 minutes in contrast with the ready destruction of exotoxin at about 60° C and a low degree of heat stability of β hemolysin Partially purified enterotoxin has been reported to have the following properties *it is a* water soluble protein of a molecular weight of 15 000 to 25 000 its iso electric *point is near* pH 8 5 it is antigenic it contains a high percentage of lysine and it is resistant to trypsin (Bergdoll 1956)

A characteristic and essentially an exclusive property of the staphylococci is their ability to clot blood plasma Clotting is brought about by *the* interaction of two factors (1) a filterable substance coagulase which is produced by the cocci and (2) a factor designated as activator or coagulase reacting factor which is normally present in the blood of man and of some animals (Smith and Hale 1944 Tager 1948) Rabbit and human plasma are readily clotted the plasma of some other animals showing considerable variation in their susceptibility to the action of coagulase The existence of several antigenically distinct coagulases is indicated by the reports of Rammelkamp and associates (1950) and Duthie (1952) Of the coagulases described by Rammelkamp coagula e I and II are distinct entities while coagulase III appears to be related to both I and II Of 4 coagulases reported by Duthie coagulases A and C are formed by human and animal strains of staphylococci coagulase B only by animal strains and coagulase D only by human strains the coagulase of essentially all human strains is inhibited by either anti A or anti D immune serum but chiefly by the former It appears likely that each of the several types of coagulase may contain some common antigens in addition to those responsible for their specific antigenic action The relationship between the coagulases described by Rammelkamp and by Duthie is not known Coagulase is formed only by pathogenic staphylococci of either human or animal origin nonpathogenic strains do not clot plasma Because of this it is possible to distinguish between pathogens and nonpathogens by a simple laboratory test Although opinion is not unanimous, it is accepted by most investigators that the coagulase test represents the most *reliable* in vitro criterion of the potential pathogenicity that is available at present Details of the test are given below (p 324)

Fibrin clots are dissolved by *an enzyme* fibrinolysin (Fisher, 1936) Lack (1948) has suggested that the responsible factor is not a true fibrinolysin but may be a staphylokinase or an activator of plasminogen Among the staphylococci the ability to dissolve fibrin clot is confined essentially to coagulase positive strains of human origin Strains which produce β hemolysin are not fibrinolytic Staphylococcal fibrinolysin is active on clots of human rabbit dog and guinea pig plasma while clots of sheep and several other species of plasma are refractory When the clot formed in plasma by a coagulase positive strain is incubated for an extended period, lysis is sometimes but not consistently obtained Lysis is best demonstrated by adding staphylococci to plasma or fibrinogen solution, which is then immediately clotted by the addition of calcium or thrombin the mixture is incubated at 37 C In contrast with the rapid fibrinolysis produced by β hemolytic streptococci often a matter of only a few minutes lysis of clots by staphylococci requires from several hours to a day or two Agar plates containing plasma have been used by some investigators to demonstrate the action of both coagulase and fibrinolysin coagulase activity is indicated during preliminary incubation by the development of a zone of opacity around the colonies which then is cleared during further incubation by the action of fibrinolysin Fibrinolysin is filterable thermostable and antigenic

The majority of strains of *Staph aureus* produce an enzyme hyaluronidase which appears to be closely related to the spreading factor described by Duran Reynals (1942) The enzyme is soluble filterable relatively heat stable and antigenic

PATHOGENESIS

Various hypotheses have been suggested concerning the possible roles of the several toxins hemolysins and enzymes in the pathogenesis of staphylococcal infections Precise information is lacking as to their functions either in permitting the cocci to become estab

the necrotic tissue are sloughed and replaced by granulation tissue The development of a carbuncle is similar but is distinguished by a lateral spreading in the deeper layers of the skin and the presence of multiple openings to the surface which discharge pus Sloughing of the necrotic tissue leaves a deep ulcer Fever and general malaise often accompany the clinical course of a carbuncle Cutaneous staphylococcal infections often tend to recur or become chronic Staphylococci are found in the majority of suppurating wounds either as the primary infecting agent or in combination with other bacteria

Invasion of the blood stream by staphylococci results in a generalized infection which assumes either of two clinical courses A fulminating systemic infection may occur which it should be emphasized is fully as severe as the septicemia due to β hemolytic streptococci It is characterized by sustained temperatures above 101 F a pulse rate of 140 or more leukocytosis and profound toxemia with symptoms of irritation of both the central *nervous system and the gastro intestinal tract* (Kleiger and Blair 1943) The course is rapid and death almost invariably occurs within a few days The more usual form of systemic infection runs a more prolonged course and is typically accompanied by the development of metastatic abscesses often multiple and commonly found in the lungs the kidneys the heart or the bones Endocarditis is a not uncommon complication while in the skin small superficial abscesses or erythematous rashes occur The responsible staphylococci may be either toxigenic or nontoxigenic While the symptoms of this form of generalized infection are often acute at the onset the temperature is usually lower than in the fulminating form or is intermittent and as the metastases develop the clinical picture becomes that of infection of the organ involved or of disturbance of a vital function Blood cultures taken preferably at the height of the fever permit differentiation of the infection from other febrile diseases *In the more severe infections* the cocci are present in the blood in fairly constant numbers they are intermittently present in variable numbers in less acute conditions The presence of staphylococci in cultures of the blood must be interpreted with caution and an attempt should be made to correlate a positive blood culture with the clinical findings The wide spread occurrence of staphylococci in the nose or on the skin increases the opportunity for contamination

Staphylococcal septicemia originates by invasion of the blood stream from a suppurative focus In the case of a cutaneous lesion anatomic features at the site of the infection sometimes determine whether or not a septicemia will develop For example should staphylococci enter the rich venous plexus in the region of the nose and the upper lip as a result of trauma to a furuncle septicemia almost invariably results Infections which follow procedures such as a cut down for intravenous therapy or cannulation may result in septicemia Systemic infection also may arise secondarily from metastatic lesions It is common in staphylococcal as in other pyogenic infections to find a suppurative phlebitis or thrombophlebitis of the major veins or of venous radicles in the immediate vicinity of a suppurative lesion arising by extension from the localized infection (Lyons 1942) The intravascular lesion appears to be *an important* point of origin of systemic infections which arise from metastatic abscesses Staphylococci may be shed into the circulation from the area of phlebitis or may be carried in fragments of infected thrombus to lodge and establish additional metastases at other sites It has been *suggested that fragments of infected thrombus* are liberated into the circulation by fibrinolysin

The mortality in systemic infections is variously reported as from 50 to 90 per cent In the fulminating type death is almost the rule A fatal outcome in the more common form of generalized infection is governed in large part by the extent of metastatic involvement of vital organs or tissues and their accessibility to surgical drainage

At least 90 per cent of all cases of osteomyelitis are caused by staphylococci In general the organisms are carried by the blood stream with or without clinical evidence of *their presence* from a cutaneous lesion to localize ultimately in the metaphyseal region of a long bone The infection is manifested clinically by the sudden onset of acute pain tenderness and local heat in the region of the metaphysis disability of the affected part and often re entry of the cocci into the circulation

Experimental infections by other bacteria or viruses are enhanced by staphylococcal spreading factor

The capacity of staphylococci to produce disease must be considered in relation to the host's susceptibility which may be affected in an appreciable degree by constitutional and other factors Although staphylococcal infections occur in persons of all ages they are particularly common in children and young adults Acute osteomyelitis almost characteristically occurs in the first 2 decades of life while some cutaneous infections coincide with physiologic changes of puberty and adolescence Conditions such as diabetes malnutrition or concurrent infection by other microorganisms tend to enhance susceptibility to the staphylococci Recent work by Schaedler Smith and Dubos indicates that rapid reversible changes in susceptibility to staphylococcal infection may be induced by nutritional and other metabolic disturbances as well as by toxemias or certain allergies

EXPERIMENTAL INFECTION

Rabbits and mice are the usual animals for studies on experimental infection or the effects of exotoxin Guinea pigs are somewhat more resistant When injected intravenously in rabbits or mice staphylococci produce an infection which usually is fatal in from one to several days Death within 24 hours often is referable to the in vivo elaboration of exotoxin When the animals survive for several days autopsy reveals metastatic abscesses chiefly in the kidneys and the heart and sometimes in other tissues, including bone A fatal infection may be produced in rabbits by the intraperitoneal injection of a virulent strain in mice this is usually accomplished only with difficulty except with certain selected strains unless the animals also receive an intraperitoneal injection of mucin

Exotoxin injected intradermally into rabbits produces an intense spreading necrosis of the skin which reaches its maximum in about 4 days and heals slowly The intravenous injection of exotoxin in rabbits or mice produces a characteristic series of events followed rapidly by death For a brief interval after injection the animal appears to be normal Then in rapid sequence it becomes unsteady and develops paralysis of the hind legs the respiration which at first is rapid becomes irregular and gasping incoordinate running movements occur there is incontinence of urine and feces Death occurs after violent convulsions or less often the animal lies passively until death supervenes The entire sequence often requires only from 1 to 5 minutes Rabbits injected intravenously with a suspension of toxigenic staphylococci which has been washed to remove any trace of free toxin die after an identical terminal reaction the only difference being that death occurs after several hours instead of a few minutes (Kleiger and Blair 1940)

The usual laboratory animals are not susceptible to staphylococcal enterotoxin However intravenous injection of enterotoxin in kittens young cats or monkeys provokes one or more bouts of vomiting and diarrhea beginning about 1 hour after its administration, the animals recover completely within 24 hours (Dack 1956) Food poisoning symptoms may be produced experimentally by feeding enterotoxin to monkeys or human volunteers the results are irregular because of individual variations in susceptibility

INFECTION IN MAN

A common site of primary staphylococcal infection in man is the skin in fact, the majority of staphylococcal septicemias and infections of the deeper tissues have their origin in cutaneous infections Among the more common infections of the skin and the subcutaneous tissues due to staphylococci are furuncles and carbuncles folliculitis sycosis paronychia and many of the common suppurative infections of wounds Staphylococci are responsible for many cases of breast abscesses and some forms of impetigo contagiosa In acne staphylococci of the *albus* variety usually are found together with a diphtheroid bacillus the latter organism being presumed to play the major etiologic role

Furuncles or boils occur singly or as multiple lesions which develop simultaneously or in successive crops Usually originating around a hair follicle a furuncle first appears as a painful indurated circumscribed area of erythema which is followed in several days by the appearance of yellow pus and softening in the center of the lesion necrosis and softening continue until eventually the pus and

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As the abscess develops the pus is under considerable pressure because of the inelasticity of the bony tissue The pus eventually finds its way to the surface of the bone where a subperiosteal abscess is formed here it may penetrate the haversian canals to infect the medullary cavity, or the soft tissue abscess breaks through to the surface of the skin Direct infection of the bone may result by extension from an adjacent lesion as occurs in osteomyelitis of the skull arising from a brain abscess Compound fractures are infected directly from the skin or dirt incident to the trauma When osteomyelitis becomes chronic as it does in many instances the local lesion often draining pus intermittently to the surface through a sinus persists for months or years with periods of quiescence broken by episodes of acute exacerbation

Staphylococcal infections of the lungs present clinical forms varying from mild tracheobronchitis to acute bronchopneumonia and the formation of multiple abscesses Approximately 9 per cent of bronchopneumonias are due to staphylococci the mortality often being high Acute staphylococcal pneumonia is assumed to originate by direct infection by cocci from the upper respiratory tract Staphylococci predominate in the friable pinkish sputum and sometimes may be isolated in essentially pure culture An intense hemorrhagic infiltration characterizes the more acute forms when less severe multiple abscesses of varying size are formed Empyema may be a complication Lowered resistance as a result of some other respiratory infection often predisposes to staphylococcal pneumonia it has been observed frequently as a complication of epidemic influenza Many instances of severe sometimes fatal, pneumonia due to antibiotic resistant strains have been encountered in recent years and have been the cause for particular concern in nurseries

An occasional complication of antibiotic therapy is the development of acute staphylococcal enteritis In the diarrheal stools of such patients staphylococci often are present in large numbers in direct gram stained preparations and antibiotic resistant *Staph aureus* usually is the predominating or the only microorganism that can be obtained in cultures It is generally assumed that the normal balance of the intestinal flora is disturbed by the antibiotic, and the staphylococci gain the ascendancy as a result of the suppression or the elimination of the normal microbial inhabitants The condition usually is relieved rapidly by discontinuing the antibiotic and substituting for it an antibiotic to which the offending strain is susceptible

Staphylococcal infections of various other tissues or organs may be encountered Paranephritic abscesses or multiple abscesses of the kidneys are common metastatic lesions In the presence of renal abscesses the urine often contains blood and yields staphylococci on culture Acute hemorrhagic nephritis is uncommon The prostate may be the site of a primary or metastatic infection Meningitis occurs secondarily to infection elsewhere in the body or as a result of direct infection of the membranes of the brain or the spinal cord following trauma Thrombophlebitis of the cavernous sinus arises secondarily to infections of the nasopharynx the nasal accessory sinuses or a furuncle of the upper lip or the nose It is accompanied by severe septicemia and may be complicated by meningitis brain abscess osteomyelitis of the skull or orbital abscess While puerperal sepsis is usually a hemolytic streptococcal infection cases due to staphylococci are encountered occasionally in rare instances the infecting staphylococcus is strictly anaerobic

Staphylococci are the most common cause of bacterial food poisoning (Dack 1956) A characteristic feature of staphylococcal food poisoning is the relatively short period of incubation The symptoms generally appear within about 1 to 6 hours after the contaminated food is eaten in contrast with incubation periods of 12 to 24 hours or more which are typical of botulism or poisoning due to the salmonella A brief period of nausea is followed by sudden paroxysms of vomiting accompanied by abdominal cramps severe diarrhea and prostration The symptoms persist for several hours the patient usually feels *quite normal 24 hours after the beginning* of the attack although weakness or nausea may persist for a day or two Fatalities are rare

The foods implicated in staphylococcal food poisoning are chiefly pastries milk and milk products and meats Pastries containing custard or cream filling are frequent offenders while a number of outbreaks have been traced

to meat or meat sandwiches The usual history is that of holding the responsible food at a relatively warm temperature for several hours before it is eaten during which time the enterotoxin is formed Most outbreaks due to milk have resulted from the consumption of raw milk

It is of some interest that the causative strains of certain clinical entities appear to be restricted to a few limited types of staphylococci Thus the predominating strains isolated from cases of staphylococcal pneumonia complicating influenza in England and Australia have been reported to be of phage type 52A or type 80 Strains of type 52A have been found by Barber to be responsible for the majority of neonatal infections in maternity units of London hospitals and also have been encountered in similar infections and in breast abscesses in this country very recently a new strain probably belonging to phage type 81, has been isolated repeatedly from these infections Most cultures of *Staph aureus* that have been incriminated in food poisoning show patterns in phage Group III while a few are lysed specifically by phage 42D (Group IV) In no case can it be considered that these strains are specific for the infections mentioned for each may be encountered in various other pathologic conditions

DEFENSE MECHANISMS

Although the skin serves as a protective barrier against infection by many bacterial species it is a common site of infection by staphylococci The healthy skin tends to free itself rapidly from organisms lodging upon it possibly because of its slightly acid reaction or certain undefined properties of the skin secretions This self sterilizing effect removes staphylococci which are transiently present but has little action on those which are permanent residents of the skin and in a significant proportion are potentially pathogenic While the pathogenesis of cutaneous staphylococcal infections is poorly understood it is assumed that some reduction in local resistance and possibly a minimal amount of trauma provide a favorable combination of circumstances for the establishment of the local lesion In the development of stitch abscesses the slight trauma and the foreign body reaction to the presence of the suture may be sufficient to permit even a minimal number of staphylococci to become established Similarly in the deeper tissues the presence of a pin a metal prosthetic apparatus or other foreign body may initiate conditions favorable to the localization of cocci that gain access to the site

The inflammatory reaction which follows the severe tissue damage caused by staphylococci tends to produce an early walling off of the infected area resulting ultimately in the circumscribed lesion which is characteristic of many staphylococcal infections This is shown experimentally by the fact that when trypan blue is injected into an area of inflammation produced as recently as 1 hour earlier by the intradermal injection of staphylococci in rabbits the dye is fixed at the site and none reaches the efferent lymphatics or tributary lymph nodes In contrast no fixation occurs for about 6 hours after the intradermal injection of Type 1 pneumococci while with β hemolytic streptococci fixation is delayed for about 2 days (Menkin 1946) The readiness with which staphylococci clot blood plasma in vitro has suggested to some that coagulase may play a role in inducing local fixation in the tissues or in the formation of thrombi Experiments by Menkin and others contradict this view

Phagocytosis by the polymorphonuclear neutrophils has long been recognized as an important mechanism in the defense against local staphylococcal infections As a part of the inflammatory response large numbers of polymorphonuclear leukocytes migrate to the site of the infection under the chemotactic influence of the cocci and of the products liberated by the tissues These are followed by macrophages the sequence of cellular changes being conditioned in part by local alterations in the hydrogen ion concentration at the site of the infection The end result of the inflammatory reaction is the formation of an abscess consisting of a central area of necrosis containing tissue debris living or dead cocci and leukocytes the whole sharply demarcated from the surrounding tissue Normally the barrier thus established effectively prevents further extension of the infection Needless to say any trauma which breaks this barrier such as squeezing a pimple or premature surgical intervention in a furuncle

may result in dissemination of the infection often with invasion of the blood stream by the cocci

When staphylococci gain access to the circulation they tend to be *removed rapidly* by the polymorphonuclear leukocytes and by the cells of the reticuloendothelial system Splanchnic removal *declines as increasing* numbers of cocci are incorporated within the leukocytes but is resumed *when extracellular* cocci reappear in the circulation It would appear that the persistence of a small proportion of viable staphylococci within the leukocytes *may prevent* their complete removal from the circulation and on occasion may result in a *low grade bacteremia* Blood serum has little or no bactericidal effect on pathogenic staphylococci (Spink and Vivino 1942 Ekstedt, 1956)

A small amount of antitoxin usually less than 1 5 International Unit is present in most normal human sera While this is insufficient to protect against infection it probably modifies the picture of superficial lesions For example Downie has pointed out that human cutaneous lesions resemble clinically and microscopically the strictly circumscribed infection which is obtained when staphylococci are injected intradermally in a rabbit immunized with staphylococcal toxoid

Skin reactions to staphylococcal filtrates are given by about 65 per cent of normal individuals and by nearly all persons with staphylococcal infections No reactions are obtained in newborn infants the proportion of positive reactors increases during the succeeding months until at the age of about 1 year the incidence of positive reactions is similar to that of normal adults Since the reaction appears to develop without relation to the antitoxin content of the blood it has been suggested that it indicates a state of allergy Type A polysaccharide produces an immediate wheal and erythema upon intradermal injection in patients with staphylococcal infection no reaction is given by Type B polysaccharide The protein common to both Type A and B staphylococci produces a delayed inflammatory reaction upon similar injection

IMMUNITY

It was pointed out above that little definite information is available of the mechanisms that are involved in the pathogenesis of staphylococcal infections In the absence of such information new studies on immunity to staphylococci would appear to lack purposeful direction, while past results are difficult of interpretation

Heat killed or formalinized vaccines stimulate the production in rabbits of agglutinins precipitins or complement fixing antibodies immune sera enhance phagocytosis but exhibit essentially no bactericidal action in vitro Following the intravenous injection of staphylococci in immunized rabbits the blood stream is cleared rapidly and may be freed permanently of cocci sterilization of the tissues is the exception although the animals may survive significantly longer than nonimmune controls (Cowan, 1939b) There is no clear relation between the presence of antibacterial antibodies and protection against the cocci there is in fact, some evidence that the observed protection is in part nonspecific

The immunization of rabbits with toxoid or toxin produces high titers of circulating antitoxin The immunity thus obtained is antitoxic and not antibacterial animals are protected against the immediate necrotic or lethal effects of the cocci or of toxin and outlive controls when challenged with a lethal intravenous dose of living cocci but metastatic abscesses ultimately develop Cutaneous lesions produced by the intradermal injection of staphylococci in toxin immune rabbits show a minimum amount of necrosis and are less extensive than those produced in controls staphylococci can be recovered in cultures from these lesions for about 1 week after injection However when rabbits are immunized with a heat killed suspension of staphylococci which has been washed to remove any trace of free toxin the intradermal injection of staphylococci results in a cutaneous lesion which is indistinguishable from that produced in controls (Downie 1937) Valentine (1936) suggested that toxoid for immunization should contain both leukocidin and exotoxin Downie's rabbits which were immunized with toxoid owed some of their immunity to antileukocidin Active phagocytosis was a prominent feature of the minimal cutaneous lesions in these animals but no phagocytosis was observed in the animals immunized with washed (toxin free) vaccine Forssman (1938) de

scribed a type of immunity which could not be ascribed to any of the known antibodies since it reached its height after agglutinins and other antibacterial antibodies had disappeared and was not related to antitoxin. Several attempts by others to reproduce Forssman's results have been unsuccessful (e.g. Downie 1937).

Following spontaneous staphylococcal infection in man the agglutinins are increased. In superficial infections the titer of circulating antitoxin rises only slightly or not at all, while in osteomyelitis it may or may not be high. A spontaneous staphylococcal infection confers little or no protection against either recurrence or subsequent infection.

Immune serum confers some degree of passive protection against experimental staphylococcal infection. Serum obtained by immunizing animals with staphylococcal vaccine has some prophylactic value when given shortly before an infecting intravenous dose of cocci but none once the infection has become established. As in the case of active immunity, the life of experimental animals may be prolonged beyond that of controls but sterilization of the tissues and the development of metastatic abscesses is not consistently obtained. Type A antibacterial serum, which precipitates the specific polysaccharides and is stated to contain no antitoxin, confers protection irregularly against cutaneous or generalized infection in animals. Antitoxic serum protects against the immediate lethal effect of toxin and against the development of cutaneous lesions in rabbits by either the cocci or the toxin. Downie's experiments on active immunity with washed vaccine and with toxoid were paralleled by similar results when sera from these animals were used for passive protection. It has been suggested that some of the protective effect of antitoxin is referable to its content of antileukocidin. Passive protection conferred by antitoxin does not prevent the development of metastatic abscesses; once the individual is enabled by antitoxin to overcome the initial period of toxicity, the clinical picture becomes that resulting from invasion of the tissues by the staphylococci.

DIAGNOSIS

The staphylococci are readily cultured from pus, blood and other pathologic material on the usual meat infusion media. When freshly isolated, colonies of pathogenic staphylococci are usually golden, varying in shade from deep gold to pale cream; less often white colonies may be encountered. For the best production of pigment the medium should contain a carbohydrate, preferably mannitol, or blood or milk. A zone of hemolysis may surround colonies on the surface of blood agar, but hemolysis does not necessarily indicate that a culture is toxigenic. The characteristic growth on agar, supplemented by the examination of Gram stained films, readily identifies a staphylococcus. Food poisoning staphylococci exhibit the same cultural reactions as other pathogenic staphylococci. Since staphylococci are often present in many foods, significance can be given to their isolation from a suspected food only when the cocci are present in very large numbers. The demonstration that the cocci from a suspected food produce enterotoxin requires the administration of culture filtrates to kittens or monkeys and is applicable only to special laboratory studies.

The selection of a suitable culture medium for the isolation of staphylococci depends upon the purpose for which the cultures are made. If, as would be the case in the diagnostic laboratory, one wishes to determine the presence of both staphylococci and such other organisms as ordinarily might be present in cultures of pathologic material, blood agar provides the most suitable medium. When one is concerned only with the demonstration of pathogenic staphylococci, as might be the case in certain epidemiologic studies, a selective medium is to be preferred. Such a medium is salt agar containing about 7 to 8 per cent of sodium chloride, which was proposed by Ludlum and has been used in various modifications by several others. This medium inhibits the growth of nonpathogenic staphylococci and many other bacteria but permits the growth of coagulase positive strains of staphylococci. Two other media may also be used to advantage: phenolphthalein phosphate agar (Barber and Kuper 1951) and tellurite glycerine agar (Zebovitz, Evans and Niven 1955). On the former medium pathogenic staphylococci are differentiated from nonpathogens by their liberation of free phenolphthalein through the action of phosphatase; the tellurite glycine agar inhibits nonpathogenic strains but permits the growth of coagulase positive staphylococci as distinct black colonies.

An important problem in laboratory diagnosis is the identification of pathogenic staphylococci and their differentiation from nonpathogens As has been mentioned above freshly isolated pathogenic staphylococci tend to exhibit pigmentation hemolysis fermentation of mannitol and liquefaction of gelatin The variations that may occur in all of these properties reduces their differential significance, especially when it is remembered that some of the same properties may be shown by nonpathogens although to a lesser degree and by a smaller proportion of strains While hemolysis has been held by some to be a criterion of pathogenicity it appears to be the experience of the majority of investigators that an appreciable number of strains from pathologic sources are nonhemolytic The term hemolytic staphylococcus has little significance in reference to pathogenicity and should be discarded

However the ability of staphylococci to clot blood plasma has generally been found to run closely parallel with the demonstrated pathogenicity of staphylococci and while opinion is not unanimous the coagulase reaction is held by the majority of workers to be the most reliable laboratory test available at present

The following technic is representative of the methods used to perform the coagulase test A young agar or broth culture is used To a serologic tube containing 0 5 cc of fresh citrated rabbit plasma diluted 1 5 with broth *is added an equal volume of a broth culture* or one loopful of growth from agar The cocci are thoroughly suspended in the plasma and the mixture is incubated in the water bath at 37° C As controls a tube inoculated with a known coagulase positive staphylococcus and a tube of uninoculated plasma are included The tubes *are examined for clotting at the end* of 3 hours Nearly all strains of pathogenic staphylococci clot plasma within this time many of them within 1 hour or less Since a small proportion of strains require a longer period of incubation tubes which show no clotting at 3 hours are incubated for an additional 18 hours The clot produced varies from one which is solid and immovable when the tube is inverted to a very loose clot suspended in the plasma Details of the technic may be varied somewhat without materially affecting the reaction Thus either citrated or oxalated rabbit or human plasma is satisfactory and the plasma may be used undiluted or diluted as much as 1 10 Plasma from the blood bank has been used for the coagulase test in some laboratories Different lots of bank plasma vary in their suitability and each lot should be checked with known coagulase positive and negative strains before use

Using the known fact that staphylococci are rapidly clumped by plasma Cadness Graves and her associates (1943) have devised a "slide test" for the identification of pathogenic staphylococci It is reliable rapid and applicable to the routine testing of cultures and to surveys in which large numbers of colonies on plates are examined directly

Cocci from a suspected colony are suspended evenly in a drop of water on a *slide* and a drop of plasma is mixed thoroughly with the suspension by continuous stirring Coagulase positive staphylococci are clumped in large irregular masses within 30 seconds or less nonpathogens are clumped only after 2 minutes or more Rapid clumping of coagulase positive staphylococci may also occur in fibrinogen solution

TREATMENT

The use of vaccines for the treatment of *chronic or recurrent* cutaneous staphylococcal infection has long been a controversial question as has been the problem whether to employ a stock or an autogenous preparation While benefit has sometimes been claimed from the use of a vaccine in individual cases it would appear that the successes of vaccine therapy are at least balanced by the failures Staphylococcal vaccines may sometimes contain a small amount of toxoid and it is not unlikely that some of their beneficial effects, both experimentally and in man may have been derived *from the antitoxic immunity* which they induced

Staphylococcal toxoid and antitoxin had their widest therapeutic use during the years just prior to the introduction of the antibiotics Although therapeutic results with both toxoid and antitoxin were promising they appear to have been discarded in favor of the antibiotics and neither agent received an extensive clinical trial Staphylococcal toxoid is of greatest value in the treatment of subacute or chronic cutaneous infections where it gen

erally has given more successful results than vaccines Clinical improvement is accompanied by an increased titer of circulating antitoxin The tissues are protected against the necrotizing effects of the toxin and normal reparative processes are enhanced but no antibacterial effect is obtained Freedom from recurrence for periods of a few months to 2 years has been claimed In approximately 3 000 cases of *cutaneous staphylococcal infection over a* period of 10 years that were treated with toxoid Ramon and his associates (1946) recorded failure in only about 7 per cent

Staphylococcal antitoxin has a definite but restricted role which is properly confined to fulminating systemic infections especially in children with evidence of severe toxemia (Kleiger and Blair 1943) When administered intravenously early and in adequate doses antitoxin may carry the patient over the acute period of emergency Upon survival of the acute phase of the infection the clinical picture becomes chiefly that of the effects of metastases to various tissues or organs Antitoxin therapy in this phase is of little value nor can it be expected to influence an infection due to a nontoxigenic strain

It is the general consensus that little is to be gained by the therapeutic use of bacteriophage When this form of therapy was used a number of years ago it appeared that localized staphylococcal infections were benefited at times but the results in septicemia were unconvincing and the mortality rate was not significantly reduced

Sulfonamide drugs sulfathiazole and sulfadiazine in particular may aid in preventing the spread of an established local infection and thus reduce the possibility of septicemia They do not appear to have prophylactic value against staphylococcal infection and on the whole are less effective in these infections than against those caused by more susceptible organisms

Among the antibiotics *penicillin* the tetracyclines and erythromycin are the most frequently used therapeutic agents in staphylococcal infections tyrothricin and bacitracin have a restricted application The ultimate therapeutic role of certain more recently developed antibiotics to which most strains of staphylococci are susceptible at present must await further evaluation The response to antibiotic therapy is governed in part by the nature and the site of the infection As a rule fresh infections in well vascularized soft tissues or localized infections which can be drained readily respond well to antibiotic therapy even in the presence of a severe systemic infection Antibiotics have proved to be a valuable adjunct in the management of surgical infections In chronic osteomyelitis the *systemic* and local administration of antibiotics permits the primary closure of a wound after adequate operative treatment and thereby reduces the chance of complications due to exogenous contamination Surgical infections due to staphylococci often exhibit collections of thick pus or lesions surrounded by relatively avascular fibrosed tissue into which antibiotics do not penetrate readily from the blood these conditions demand that the systemic administration of antibiotics be supplemented by their local injection and frequently by surgical treatment It cannot be emphasized too strongly that in any condition in which sound surgical judgment indicates the necessity for operation the complementary use of antibiotics does not alter the general principles of surgery Antimicrobial drugs provide a useful aid to surgery but do not supplant adequate surgical intervention Failure of antibiotic therapy may be traced to such factors as inadequate dosage overwhelming infection use of the agent in terminal stages of the infection inadequate surgical treatment (sometimes unavoidable because of the anatomic site of the lesion) or infection by a strain which is not susceptible to the drug being administered Mixed infections with gram negative bacilli present especially difficult therapeutic problems

In a majority of cases the administration of a single antibiotic is effective In the presence of a resistant infection it often may be necessary to change to an antibiotic to which the organism is known to be susceptible While *empirical treatment ometimes is required at* the beginning of a severe infection it is becoming increasingly important in staphylococcal infections to obtain a culture of the blood or from the lesion as early as possible for the determination of antibiotic sensitivity The use of 2 antibiotics in combination sometimes provides an enhanced therapeutic effect which cannot be obtained by the use of either anti

biotic alone or when the dose of a single antibiotic cannot be increased

When the responsible organisms are sensitive to penicillin this antibiotic still remains the drug of choice provided that careful attention is paid to adequate dosage proper duration of treatment and the selection of suitable cases

Tyrothricin or bacitracin may be used topically but the possibility of toxic reactions contraindicates their administration systemically Under special circumstances bacitracin must be administered systemically its use then is reserved for those infections in which the responsible staphylococcus is resistant to all other antibiotics

In the treatment of staphylococcal infections whether of a medical or a surgical nature attention should be paid of course to any necessary supportive therapy such as for example the control of anemia by the use of blood transfusions the maintenance of proper fluid balance and the control of diabetes or other concurrent debilitating disease

EPIDEMIOLOGY AND CONTROL

The wide distribution of staphylococci and their natural parasitic association with man suggest that the opportunities for exposure to pathogenic strains are numerous However the actual incidence of clinical infection is lower than might be expected from the known ubiquity of the organisms This probably can be attributed in part to large variations among individual strains in their capacity to initiate disease coupled with differences in the susceptibility of the potential hosts to infection

In the hospital several factors combine to provide considerable opportunity for exposure to staphylococci particularly to antibiotic resistant strains It is now a common experience that the incidence of antibiotic resistant staphylococci isolated in the hospital is high In addition to their occurrence in most cross infections resistant strains also are harbored in the nasopharynx of a large proportion of healthy carriers among the nursing and the medical staffs and may be acquired by the patients after a certain period of hospitalization The incidence and the distribution of strains among the several phage groups has been found to be roughly parallel in both personnel and patients (Jackson, Dowling and Lepper, 1954b) In the presence of an unusual number of infections or an epidemic due to a strain of a given phage or serologic type for example in a nursery, the same strain usually is harbored by the majority of the carriers and may also be found in the environment When staphylococci are shed into the immediate vicinity of an infected individual to lodge on dressings or bedding or to find their way ultimately into the dust or the air their chance of survival is enhanced by their relative resistance to drying or other adverse environmental conditions

It is debatable whether staphylococci have undergone an actual increase of virulence in recent years While some evidence has been presented in support of this view it is difficult if not impossible to compare accurately the virulence of strains now being encountered with that of strains isolated before the advent of antibiotics The apparent prevalence of neonatal infections severe and sometimes fatal pneumonia and some other conditions due to staphylococci may be an expression of the emergence of antibiotic resistant strains in a field that has been prepared by the elimination of sensitive species It is generally recognized that because of their immediate past history of having produced disease staphylococci from an active lesion are potentially more dangerous than those harbored by a healthy carrier However one cannot summarily dismiss as innocuous every coagulase positive staphylococcus that occurs in the healthy nose it has been shown experimentally that some strains from the nose can produce a purulent lesion in the human skin when local conditions at the site of injection permit multiplication of the cocci

The chance of acquiring antibiotic resistant staphylococci increases with the length of stay in the hospital (Barber and Whitehead 1949) It is greater in those who are hospitalized for 2 weeks or more than in those who remain in the hospital for 1 week or less In student nurses the rate of carriage of resistant staphylococci remains low during the initial period of classroom instruction and rises soon after they enter the wards for training

The fact that many of the patients who are assembled in the hospital are poorly equipped to withstand infection by reason of the disease or the debilitating condition for which

they are admitted increases the possibility that infection may develop when they are exposed to pathogenic staphylococci Without denying their unquestioned benefits some modern therapeutic procedures such as irradiation or the administration of steroids and certain other agents occasionally promote an incipient infection The frequent use of venipuncture cannulation or skin puncture in current practice requires that these procedures be performed in the already susceptible individual with extraordinary precautions against infection

It is essentially a universal observation that the majority of antibiotic resistant staphylococci isolated in the hospital fall into the broad phage Group III Next in order of frequency of resistant strains are those of Group I while resistant strains belonging to the other groups appear to be significantly fewer The evidence suggests that with the gradual elimination of sensitive strains by antibiotics there has become established in the hospital community a reservoir composed of a relatively limited number of antibiotic resistant strains which serves as a constant potential source of cross infection (Rountree and Thomson 1949)

The incidence of carriers of coagulase positive staphylococci in the general population has been variously reported to be from about 30 to 50 per cent (Gillespie Devanish and Cowan 1939) Without doubt many factors which contribute to the risk of exposure within the confines of the hospital also exist in the general population Outside of the hospital however the chance of exposure to pathogenic staphylococci would appear to be somewhat reduced by the factor of dilution and it would not appear that the opportunity for exposure has changed materially over the past several years The incidence of antibiotic resistant staphylococci in the population at large has continued to remain low ever since antibiotics were first used therapeutically about 15 years ago However a possible portent of the future is found in evidence supplied by phage typing that the individual with a hospital acquired resistant strain may transmit the cocci to some members of his immediate family who in turn can become carriers or even victims of staphylococcal disease (Jackson Dowling and Lepper 1954b)

Attempts to control hospital cross infections involve the application of measures designed to prevent the dissemination of staphylococci from patient to personnel and vice versa and to avoid dispersal of infective material into the immediate surroundings Strict attention to the principles of asepsis and antisepsis is required both in the operating room and in the wards including adequate masking good dressing technics and the careful performance of venipuncture and skin puncture The employment of modern methods of dust suppression and of treatment of bedding must be considered while contaminated dressings and other infective material should be destroyed immediately or otherwise rendered harmless While it often is not practicable to isolate the patient some degree of isolation of a superficial lesion may be accomplished by the use of an occlusive dressing Attempts to render a persistent carrier free from the offending cocci by a suitable course of antibiotics usually have not been notably successful In special cases it sometimes has been necessary to transfer a persistent carrier to a position which did not require direct contact with the patients The prevention of cross infection is not easy and its attainment demands the conscientious efforts of every individual concerned

In cases of food poisoning the incriminated food often is found to have been prepared or handled by an individual who is a nasal or skin carrier of pathogenic staphylococci or has an open staphylococcal lesion on the hands the arms or the face In several epidemiologic studies phage typing has demonstrated the identity of the strain isolated from the food with that carried by the food handler in certain instances in which milk was implicated as a cause of food poisoning the strain has been traced similarly to a cow or a goat suffering from staphylococcal mastitis Reports of outbreaks of staphylococcal food poisoning show that not infrequently after the food has been prepared it is held at room temperature for several hours before being served inadvertently providing a period of incubation during which the enterotoxin is formed The ubiquity of staphylococcal carriers makes it impracticable to eliminate from food handling

every person with staphylococci on the skin or in the nose Of course, individuals with obvious cutaneous infections should be excluded from the preparation or handling of food The simplest and most reliable method of control involves the refrigeration of all food substances which may be a potential source of food poisoning The principle of pasteurization has been applied to the control of pastries reheating cream puffs or eclairs for a brief period after they have been filled when properly carried out the treatment is said not to affect the flavor or the appearance of the pastry

GAFFKYA TETRAGENA

These organisms occur as spherical cells and typically are found in body fluids as groups of 4 They are gram positive facultatively anaerobic and grow slowly on artificial media They are parasitic upon the mucous membranes of the respiratory tract The type species is *Gaffkya tetragena* (Gaffky) Trevisan

The distinguishing feature of the cocci is their arrangement in tetrads While this grouping is always seen in material taken directly from the body the cocci in cultures usually are arranged in 4 s or pairs In pus or body fluids the tetrads are surrounded by a wide capsulelike halo which is not always demonstrable in cultures The diameter of the individual cocci is about 0 8 micron On agar the colonies are grayish white less opaque and somewhat smaller than those of the staphylococci and are adherent and viscid Variants occur which show smooth or mucoid yellow pink or brown colonies Several carbohydrates are fermented milk is coagulated and gelatin is not liquefied Originally known as *Micrococcus tetragenus* these organisms form a separate genus of the family *Micrococcaceae* (Bergey s Manual 1957)

The pathogenicity of *Gaffkya tetragena* for mice is high and is characterized by a rapidly fatal septicemia with metastases in various organs Rabbits and guinea pigs are less susceptible and often show only a localized lesion

Relatively few human infections due to *Gaffkya tetragena* have been reported, although it is possible that they are more common than has been suspected It would appear that infection takes place only when the host s resistance is lowered by some predisposing condition, a common precursor is a respiratory infection Young adults appear to be particularly susceptible Abscesses of the soft tissues arthritis meningitis pneumonia empyema and endocarditis have been described and a penile ulcer due to an anaerobic form has been reported The course of infection may range from mild to severe and is accompanied by a remittent fever and leukocytosis Several instances of septicemia have been reported some of which were fatal

Treatment with vaccine has given variable results The successful use of penicillin in sepsis due to *Gaffkya tetragena* has been reported

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The Anthrax Bacillus

Anthrax is primarily an acute infectious disease of domestic herbivorous animals the disease in man is contracted by contact with infected animals or contaminated animal products *Bacillus anthracis* the causative agent is a large gram positive rod shaped organism which forms resistant spores and except for its property of virulence is not unlike saprophytic members of the genus

HISTORY

The disease has been known from antiquity and satisfactory descriptions of the typical lesions in animals and man have been available for about 200 years *B anthracis* was the first micro organism to be established as the etiologic agent of a disease It was discovered in the blood of infected sheep by Rayer in 1850 and experimental transmission of the disease was demonstrated by Davaine in 1863 Koch (1877) described the isolation and the cultivation of the organism the formation of spores the production of the disease in experimental animals and the recovery of the organism from the experimental infection These classic experiments represented a major advance in the understanding of anthrax and provided the basic concepts and methods for the rapid expansion of medical bacteriology Immunization of animals with attenuated living vaccines was introduced by Pasteur and a field trial at Pouilly le Fort in 1881 established the effectiveness of the procedure The subsequent introduction of the vaccines into general use provided a major stimulus for early investigations in immunology

MORPHOLOGY AND CULTURAL CHARACTERISTICS

Bacillus anthracis is a large gram positive, spore forming rod 1 to 1 5 μ in width and *4 to 8 μ in length Large capsules are formed* in vivo and under appropriate cultural conditions in vitro Spores are not formed in the living animal In smears from an infected host the organisms lie singly or in short chains capsules are formed and may be demonstrated by Giemsa or similar methods of staining The appearance of organisms in artificial media is considerably different In young cultures on nutrient agar the bacilli are non encapsulated rods with square ends arranged in strands of long chains Under aerobic conditions sporulation begins toward the end of logarithmic growth and is usually well advanced after 48 hours of incubation Spores are oval in shape and are formed equatorially They are liberated by autolysis of the vegetative cells pairs or short chains of spores may remain connected by linkages which are evidently extensions of the spore coat (Roth et al 1956) The organism is nonmotile The motile strains that have been described were presumably erroneously identified and the reported induction of motility by bacteriophage (Brown et al 1955) has not been confirmed by further investigation (Cherry 1957)

Typical colonies of virulent wild type strains that have grown 24 hours on a medium such as nutrient agar are flat dull gray and *medusa head in appearance The last char*



FIG 30 Typical lesion of cutaneous anthrax 3 to 4 days old The black center is surrounded by a ring of vesicles painless edema encircles the lesion (Cold 1955)

acteristic is caused by outgrowths which radiate from the margin of the colony and then curve back toward it so that the colony appears to be spinning The organism is nonhemolytic or only slightly hemolytic on blood agar and this characteristic serves to differentiate it from many saprophytic bacilli When grown in the presence of carbon dioxide the organism are encapsulated and produce colonies that are round and mucoid (Thorne 1956)

The organism grows well on nutrient agar and other general media In chemically defined media the requirements for rapid growth are rather complex and include thiamine and a considerable number of amino acids in addition to inorganic salts (Proom and Knight 1955) Complex interrelationships with respect to amino acid requirements now recognized in many organisms were first demonstrated with *B anthracis* (Gladstone 1939) Purines and pyrimidines stimulate growth of many strains Although molecular oxygen is necessary for sporulation germination of spores occurs under anaerobic as well as aerobic conditions (Roth and Lively 1956) Rapid germination of spores occurs in the presence of adenosine L alanine and L tyrosine Adenosine may be replaced by certain related ribosides the activity of L alanine is antagonized markedly by D alanine (Hills 1950) The organism grows best aerobically but growth also occurs under strict anaerobic conditions Acid without gas is produced in the presence of glucose levulose sucrose maltose trehalose and dextrin Mannose and the pentoses are not fermented The organism produces a typical heterolactic fermentation of glucose acetic succinic lactic and formic are



FIG 31 Colony of virulent *B anthracis* grown on nutrient agar and stained with methylene blue (A) The whole colony × 45 (B D) Border of the same colony × 145 × 400 and × 1 600 respectively Note the parallel arrangement of the bacterial filaments (Stein C D Anthrax in animals and its relationship to the disease in man Ann New York Acad Sc 48 507 534)

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The Anthrax Bacillus

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FIG 32 Colonies of virulent *B anthracis* and of an avirulent nonencapsulated mutant grown on bicarbonate containing agar in the presence of carbon dioxide The mucoid colonies of the virulent strain may be distinguished readily from the flat rough colonies of the nonencapsulated mutant (Thorne C B Ann New York Acad Sc vol 48 Art 6)

the principal acids formed (Puziss and Rittenberg 1957) The fermentation is not unlike that carried out by saprophytic members of the genus Acetylmethylcarbinol glycerol and 2 3 butylene glycol are produced nitrates are reduced to nitrites and gelatin is slowly liquefied Protease amylase catalase lecithinase collagenase and D and L amino acid transaminase may be demonstrated in whole cells homogenates or culture filtrates Certain strains become susceptible to the action of lysozyme when grown in the presence of carbon dioxide (Gladstone and Johnston, 1955)

VARIATION

The foregoing description is applicable to typical virulent strains as isolated from the field Variation occurs among wild type strains in characteristics such as virulence, nutritional requirements and sensitivity to bacteriophage and lysozyme In addition mutants are readily obtained under appropriate conditions of cultivation in the laboratory Serial transfer on laboratory media particularly at elevated temperature leads to a gradual decrease in virulence Since the work of Pasteur strains attenuated in this manner have been used for preparation of living vaccines The colonies of the attenuated vaccine strains are smoother and more dome shaped than virulent strains and the characteristic marginal projections are absent The attenuated strains have acquired the ability to form capsular material when grown in air on nutrient agar Overgrowth of virulent cells by avirulent mutants occurs slowly and attenuation is a gradual process (Preisz, 1911) Nonsporulating mutants may appear particularly during cultivation in the presence of relatively high concentrations of calcium salts which inhibit sporulation and favor the emergence of nonsporulating mutants (Renaux, 1952) Nonsporulating mutants may be virulent or avirulent indicating that sporulation and virulence are independent characteristics

The colonies of virulent strains are rough when grown on nutrient agar and the relatively smooth growth of the attenuated strains has led to the misconception that *B anthracis* represents an exception to the rule that loss of virulence accompanies the S→R variation However the fully virulent organisms form large capsules in vivo and also during growth in vitro in the presence of carbon dioxide Under the latter conditions the colonies are mucoid in appearance indicating that the fully virulent strains are not rough if cultivated under appropriate nutritional conditions After about 48 hours of incubation small rough outgrowths appear on the margin of the colonies and careful selection from these areas yields mutants that are rough under all conditions of cultivation and have lost the ability to synthesize the capsular polypeptide (Sterne 1937 Thorne 1956) These avirulent mutants have found increasing u e as living vaccines

Additional colonial variants that arise in old cultures were described by Nungester (1929) These include avirulent mucoid mutants that are heavily encapsulated when grown on nutrient agar in air several types of smooth colonies and phantom colonies that grow as a thin film on the surface of the medium Nonproteolytic mutants have been isolated virulence appears to be unchanged by this mutation (Wright et al 1951, and unpublished data) Little information is available regarding the enzymatic alterations associated with these mutations

ISOLATION AND IDENTIFICATION

Isolation of *B anthracis* presents no problem if the specimen contains an appreciable number of the organisms and is fresh and not excessively contaminated Nutrient agar blood agar and egg yolk agar have been recommended for primary isolation Blood agar has the advantage that many colonies otherwise resembling those of *B anthracis* may be excluded by their strong hemolytic activity Typical colonies may be selected and identified by cultural methods and virulence tests The organism is rapidly killed by putrefactive processes and cultural methods may yield negative results with specimens from decomposed carcasses Inoculation of mice or guinea pigs may allow primary isolation when cultural methods are unsuccessful but contaminating organisms particularly *Clostridia* may cause rapid death of the animals In these cases superficial inoculation by application of the suspension to the scarified skin may prove to be successful Isolation of spores from heavily contaminated samples such as soil manure bone or hair may be more difficult and methods must be selected for each specimen In general the material should be suspended in saline and heated at 65 C for 10 minutes to destroy vegetative organisms before culture or animal inoculation Selective media have been described by Pearce and Powell (1951) and by Morris (1955)

Identification of *B anthracis* is usually a problem of differentiation from *B cereus* and occasionally from *B megaterium* or *B subtilis* For tentative identification reliance may be placed on colonial and microscopic morphology the virtual absence of hemolytic activity on sheep blood agar and the absence of motility Supporting evidence may consist of sensitivity to 10 units/ml of penicillin ability to form capsules in the presence of 20 per cent carbon dioxide but not in air and susceptibility to bacteriophage (McCloy 1951 Brown and Cherry 1955) Pathogenicity for mice or guinea pigs may be used for final confirmation Small inocula should be used for this purpose because saprophytic members of the genus may produce fatal infection or intoxication if inoculated in large numbers especially intraperitoneally Slow or negligible fermentation of salicin slow reduction of methylene blue and slow liquefaction of gelatin are additional characteristics of *B anthracis* that may aid in identification (Stein 1944)

PATHOGENICITY

Virtually all animals are in some degree susceptible to infection with *B anthracis* In nature or under the conditions of normal animal husbandry infections have occurred in cattle sheep horses goats buffaloes water buffaloes minks swine deer ostriches and elephants Outbreaks occur occasionally in zoos and many species of carnivorous animals and birds have been infected under these circumstances by ingestion of contaminated meat (Stein and Van Ness 1955) Experimental infections have been produced in mice rats guinea pigs rabbits and many wild rodents monkeys and even frogs and fish Susceptibility varies greatly among different species and strains Of the common laboratory animals mice and guinea pigs are the most susceptible to challenge by the cutaneous route (LD_{50} less than 5 spores) Rabbits goats and sheep are also highly susceptible White rats cats dogs cattle and swine are much less susceptible and usually survive subcutaneous injection of 10^8 spores although a localized infection may occur at the site of inoculation The susceptibility of a species may vary with age and may be altered experimentally by changes in body temperature by administration of certain hormones or by preexisting infection with unrelated organisms

Cutaneous anthrax or malignant pustule is the most common form of the disease in man Frequently the infection develops at the site of a negligible injury such as a scratch or a minor abrasion The organism is apparently unable to penetrate the intact skin Cutaneous lesions occur almost exclusively on exposed portions of the body *more than 95 per cent* occur on the head the neck and the upper extremity (Legge 1934) The evolution of the cutaneous lesion is described in the section on diagnosis Before the advent of antibiotic therapy the mortality of cutaneous anthrax over a 20 year period in the United States was 21 per cent of the 1 683 reported cases (Smyth 1941) However the true mortality of untreated human anthrax is difficult to estimate because failure to report less

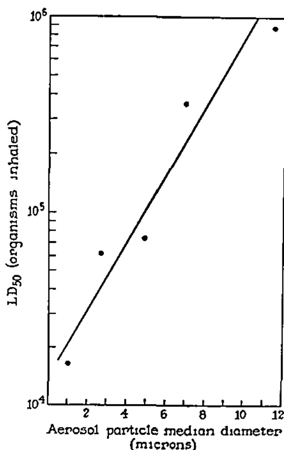


FIG 33 Effect of aerosol particle size on respiratory virulence of *Bacillus anthracis* for guinea pigs (Data of W C Day and R J Goodlow Presented at Annual Meeting Soc of Am Bacteriologists 1957)

severe cases tends to raise the apparent mortality whereas therapeutic measures doubtless reduced the mortality It is evident that in the absence of therapy an appreciable proportion of cutaneous infections in man would progress to generalized infection and death Meningitis due to *B anthracis* is a relatively rare and almost invariably fatal manifestation of human anthrax the disease is usually a complication of primary infection elsewhere in the body (Haight 1952)

Infection may occur not only by the cutaneous route but also via the respiratory and the alimentary tracts and experimentally via the intramuscular the intraperitoneal and the intravenous routes of inoculation Although there is no evidence that the respiratory route is of importance in pathogenesis of the natural disease in animals experimental inhalation anthrax has received considerable study since the original investigations of Buchner in 1888 In the guinea pig spores deposited in the alveoli are taken up by alveolar phagocytes and carried to the tracheobronchial lymph glands Here the bacilli that escape destruction by the normal defensive mechanisms of the host multiply rapidly the infection spreads via the efferent lymphatics to the circulation (Ross 1957) The LD_{50} of anthrax spores for susceptible animals such as guinea pigs and monkeys is approximately 20 000 organisms the size of the dose suggests that the clearing mechanisms of the lung are usually effective and only the occasional spore is able to initiate progressive infection Many of the spores deposited in the lung have been found to remain inactive for long periods Monkeys challenged by the respiratory route and protected by daily administration of penicillin died of anthrax when the drug was discontinued after treatment for 10 days Viable spores could be detected in the lungs of immunized monkeys 100 days after respiratory challenge (Henderson et al 1956)

The infectivity of aerosols for guinea pigs and monkeys is influenced markedly by particle size (Druett et al 1953) Particles less than 5 microns in diameter are much more likely to penetrate into the alveoli and be deposited on the alveolar wall (Harper and Morton 1953) Representative data on the influence of particle size on the LD_{50} of *B anthracis* spores is presented in Figure 33 It may be noted that the LD_{50} increases markedly as the particle size is increased from 1 to 12 microns Certain chemical compounds when added to aerosols of spores significantly reduce the infectious dose (Young and Zelle 1946 Barnes, 1947)

Inhalation anthrax of man (Woolsorter's disease) was formerly a relatively common form of industrial anthrax Improvements in working conditions in industries that deal with contaminated hair and hides have greatly reduced the exposure of workers to spore bearing dust and presumably for this reason the incidence of inhalation anthrax has declined markedly Practically the entire respiratory tract is susceptible to infection but the bronchi and the lungs are most frequently the site of the primary lesion The mortality of diagnosed cases approaches 100 per cent

although it is possible that nonfatal cases escape diagnosis The acute course of the disease is not referable to the pneumonic lesions which may be minimal but rather to the rapid generalization of the infection via the lymphatics to the circulation

Fatal infection of cattle and severe infection of swine may be produced by the oral route although these animals are resistant to cutaneous challenge (Schlingman et al 1956) Dogs sheep and many other animals may be infected by feeding however guinea pigs and rabbits are not infected by oral administration of 10^9 spores (Druett et al 1953) It is evident that susceptibility by different routes of infection may vary independently and that the infective dose by one route of challenge gives no indication of susceptibility by another route Human infection by the oral route is of negligible importance in civilized countries but significant outbreaks with a high mortality occur in primitive societies in which meat from infected animals is used for human food Additional information is needed regarding the infective dose and mortality in such outbreaks

No direct information is available regarding the infective dose for man by the various routes and estimates of human susceptibility must be inferred from indirect evidence The significant mortality of cutaneous anthrax indicates that man is more susceptible than white rats cattle and swine The tissue reaction to infection and the occurrence of spontaneous recovery from cutaneous infection suggest that man is less susceptible than the guinea pig the rabbit and the monkey Susceptibility by the respiratory or by the oral route are even more difficult to estimate Only rarely do workers contract recognized inhalation anthrax in environments in which spores may be isolated from the nose and the throat (Carr and Rew 1957) however it is possible that the spore bearing particles in these situations are relatively large There is no evidence that man is less susceptible to small particle aerosols than the guinea pig and the monkey

FACTORS IN INFECTION AND RESISTANCE

Anthrax has long occupied a favored position as a model infection for investigation of mechanisms of infection and resistance This has resulted from the acute course of the infection with its dramatic terminal bacteremia the wide variation in the susceptibility of different species, and the economic and medical importance of the disease Early investigations have been reviewed by Sobernheim (1931) and Eurich and Hewlett (1930) and more recent work by Smith and Keppie (1955) and by Sterne (1958) Organisms injected into highly susceptible animals proliferate freely and remain fully encapsulated few leukocytes accumulate and rapid generalization of the infection occurs In less susceptible animals the course of the infection is similar for the first few hours After this period however large numbers of leukocytes appear in the lesion and the organisms lose their capsules and disintegrate without conspicuous phagocytosis (Cromartie et al 1947) During growth in vivo the organism produces aggressins which act to overcome the normal defensive mechanisms of the host Study of fractions of infected tissue and of organisms grown in vivo suggest that at least 3 substances contribute to aggressin activity the glutamyl polypeptide of the capsule the extracellular protective antigen and an intracellular anticomplementary substance (Smith and Keppie 1955) These substances inhibit the bactericidal or phagocytic activities of the host and permit progressive infection The bactericidal activity of serum leukocytes and tissue extracts is evidently due to the presence of basic polypeptides containing lysine The polypeptide causes a rapid inhibition of in vitro oxygen uptake by suspensions of *B anthracis* presumably its protective activity in vivo is exerted in a similar manner (Bloom et al 1947 Bloom and Prigmore 1952) The course of the infection is determined to a considerable degree by the relative ability of the organism to elaborate aggressins and the host to mobilize bactericidal substances The encapsulated bacilli are resistant to the phagocytic cells of relatively resistant as well as susceptible animals and it is probable that phagocytosis is of minor importance in natural immunity to infection with virulent strains

The mechanism of death in anthrax has aroused the interest of investigators for many years Sterile extracts of infected tissue were shown to have edema producing activity in the skin of normal animals but lethal activity

could not be demonstrated Culture filtrates organisms grown in vitro or in vivo and killed in various ways and extracts of such organisms likewise exhibited no significant toxic activity It seemed possible that the massive terminal bacteremia that occurs in the guinea pig and certain other species was directly responsible for death Blockage of capillaries or exhaustion of oxygen or of essential host nutrients were suggested as basic mechanisms of death in the infection These theories were rendered untenable and the problem was greatly clarified by the recent investigations of Smith Keppie and associates (see Smith and Keppie 1955) Treatment of infected guinea pigs with streptomycin promptly terminated the infection even when the drug was administered a few hours before death would otherwise have occurred Multiplication of the organisms ceased and the bacteremia rapidly disappeared However, when the bacteremia had exceeded 3×10^6 chains of organisms per ml of blood the animals died of anthrax despite complete clearance of organisms from the circulation The critical stage was reached at a point only 1/300th the level of the bacteremia at death in untreated animals These experiments demonstrated that a factor other than the physical presence of the organisms in the circulation was of critical importance and again attention was directed to other mechanisms and to search for a lethal toxin

Detailed study of infected guinea pigs revealed that in the terminal phases the animals presented a classic syndrome of secondary shock The manifestations included reduced blood volume and pressure hemoconcentration the presence of edema and hemorrhage and fall in body temperature Disturbances in electrolyte balance and in phosphate and carbohydrate metabolism were prominent and there was evidence of acute renal failure A renewed search was made for the factor responsible for these effects It was found that sterile heparinized plasma of guinea pigs dying or near death from anthrax was capable of producing extensive edema in the skin of normal guinea pigs and the material was lethal to mice and guinea pigs when injected intravenously or intraperitoneally The toxicity was specifically neutralized by anthrax antiserum Presumably, the plasma toxin is closely related to the tissue damaging factor that has been detected in extracts of anthrax lesions, small amounts of toxin evidently re produce the effects obtained with the extract The toxin was separated by ultracentrifugation into two fractions, both of which were necessary for full activity One fraction appears to be related to protective antigen produced in vitro (Smith et al 1956a) The toxin is also elaborated during in vitro growth of the organism in heparinized or defibrinated guinea pig blood at 37° C (Harris Smith et al 1957) The toxicity appears in very young cultures and is destroyed rapidly after about 5 hours of incubation

ANTIGENIC STRUCTURE

Three distinct antigens are well recognized the protective antigen the capsular polypeptide and the somatic polysaccharide Protective antigen is the substance responsible for development of immunity to infection in most animals Bail in 1904 demonstrated this antigen in edema fluid of anthrax lesions and these observations have been confirmed and extended by subsequent investigators However protective antigen remained uncharacterized and attempts to produce it in vitro were unsuccessful Gladstone (1946) demonstrated elaboration of the antigen during growth of the organism in whole serum under rather critical conditions Subsequently, Wright et al (1954) obtained elaboration of protective antigen in chemically defined non protein media and these observations have been confirmed and extended by Belton and Strange (1954) Puziss and Wright (1954) and Thorne and Belton (1957) The antigen is evidently a protein partial purification from culture filtrate was reported by Strange and Belton (1954) Elaboration of the antigen occurs only under particular cultural conditions one of the most consistent nutritional requirements is for bicarbonate ion (Puziss and Wright 1954) The antigen is found in the culture filtrate rather than in the bacterial cells and is elaborated during anaerobic as well as aerobic growth (Wright and Puziss 1957)

Attempts to demonstrate in vitro serologic reactions with protective antigen in crude preparations such as edema fluid or serum culture filtrates were unsuccessful Accord

ingly protective antigen was detected and quantified by means of its immunizing activity in rabbits or guinea pigs Recently more precise and convenient methods for estimation of protective antigens have been proposed Belton and Henderson (1956) have described an in vivo titration procedure based on neutralization by protective antigen of the anti toxic activity of anti era for the in vivo toxin of Smith et al McGann and Jones (1958) have devised a complement fixation titration the results of which correlate in general with the immunizing activity of different preparations of antigen The agar diffusion precipitin method of Ouchterlony reveals a line of precipitation whose presence and intensity evidently correlate with immunizing activity (Thorne and Belton 1957) Further investigation will be required to confirm and establish the specificity of these procedures

The principal component of the capsule is a polypeptide of glutamic acid This substance plays a major role in virulence of the organism since nonencapsulated mutants which do not elaborate polypeptide are avirulent The polypeptide has aggressin activity and because of its concentration around the organism it probably exerts a considerable effect in preventing phagocytosis (Smith and Keppie 1955) Precipitating antisera reactive with the polypeptide may be prepared by immunizing animals with encapsulated organisms the isolated polypeptide reacts with these antisera but is not itself antigenic Capsular antibody does not display protective activity except in the mouse (Ivanovics 1938)

Polypeptides that are similar chemically and related antigenically are elaborated by *B subtilis* and by certain other *Bacilli* The *anthracis* polypeptide is composed entirely of *D* glutamic acid the polypeptide elaborated by *B subtilis* contains also *L*-glutamic acid the proportion varying with cultural conditions The amino acid residues are connected primarily if not exclusively by gamma linkages The chemical structure and mechanism of synthesis of glutamyl polypeptide has been reviewed by Thorne (1956)

The somatic polysaccharide contains equimolar quantities of D glucosamine D galactose and acetic acid (Ivanovics 1940a) The structure of the polysaccharide has been elucidated by Mester and Ivanovics (1957) A small peptide moiety containing α ϵ diaminopimelic acid is closely associated with the polysaccharide and the complex appears to form part of the cell wall (Smith et al 1956b) The polysaccharide reacts in high dilution with antianthrax serum and cross reacts with the polysaccharide of type 14 pneumococcus and with partially hydrolyzed blood group A substance (Ivanovics 1940b) The polysaccharide is devoid of aggressin activity and evidently plays no important role in virulence

In addition to these relatively well characterized antigens a phosphorylated mannan that reacts with rabbit but not with horse antianthrax serum has been isolated by Cave Browne Cave et al (1954) Additional antigens doubtless await isolation and characterization

ACQUIRED RESISTANCE AND IMMUNIZATION

The observation that animals that survive anthrax become resistant to reinfection has been amply confirmed since the initial report of Chauveau in 1880 Second attacks of human anthrax are rare but apparently authentic instances have been reported Vaccines composed of suspensions of killed organisms produce no significant increase in resistance Pasteur introduced the use of attenuated strains for vaccination of dome tic animals in 1881 and this method with subsequent modifications and improvements has been widely used where anthrax is a significant economic problem The organism was attenuated by growth at 42 to 43 C Originally two vaccines of different degrees of attenuation were used the first was virulent for mice but avirulent for guinea pigs and rabbits and the second was virulent for guinea pigs and some rabbits The use of two vaccines was gradually discarded and a single vaccine virulent for guinea pigs but not for rabbits came into general use Saponin is usually added and in proper concentration increases the antigenicity of the preparation

In practice it has proved to be difficult to maintain the virulence of the Pasteurian vaccines at the proper level If the vaccine is too virulent excessive losses occur among vaccinated animals If the virulence is insufficient the vaccine is unable to produce the degree

of infection that is necessary for development of immunity This difficulty was overcome by the introduction of nonencapsulated spore vaccines by Sterne (1939) The nonencapsulated mutants have lost the ability to form the glutamyl polypeptide and are essentially avirulent They sporulate well, and vaccines are readily prepared on a large scale These vaccines produce smaller losses among vaccinated animals than Pasteurian vaccines, and a single preparation is suitable for all species of animals They were first introduced and used on a large scale in South Africa and are replacing the Pasteurian vaccines elsewhere None of the living spore vaccines is generally *considered safe for immunization of man*

For many years attempts have been made to develop effective nonliving antigens Until recently none of the antigens produced in vitro contained significant protective antigen and their immunizing effectiveness was negligible The work of Gladstone and subsequent investigators in developing conditions for elaboration of the protective antigen has been described above Alum precipitated preparations of protective antigen elaborated in vitro have been shown to produce effective immunity in guinea pigs rabbits monkeys sheep and cattle (Wright et al 1954 Schlingman et al 1956 Darlow et al 1956) The antigen has been used in man without serious local or systemic reactions clinical trial in *occupationally exposed workers is in progress* The preparations have not reached a stage of development at which they can replace spore vaccine for large scale immunization of cattle and other animals

DIAGNOSIS

Prompt and accurate diganosis of cutaneous anthrax in man usually presents no serious difficulty The characteristic lesion begins as a small red macule which enlarges and local edema of varying extent gradually develops in the surrounding tissue *A vesicle filled* with clear fluid occupies a central position and is soon followed by satellite vesicles The organism may be easily demonstrated in the early lesion by cultural methods and may frequently be detected in stained smears Fluid and scrapings from the base of a previously unopened vesicle should be used for preparation of slides and of cultures on nutrient or blood agar Microscopic examination of Gram or Giemsa stained films will frequently reveal the organism and permit a rapid tentative diagnosis The lesion gradually becomes necrotic, with evolution of the black eschar characteristic of anthrax At this stage demonstration of the organism is less certain and secondary infection may complicate the bacteriologic diagnosis Occasionally inoculation of mice or guinea pigs will allow isolation of the organism when direct cultural methods fail Antibiotic therapy usually causes rapid disappearance of the organism, even thou h evolution of the local lesion continues Therefore specimens for bacteriologic study should *be obtained before therapy is initiated*

Extension to the regional lymph nodes and to the blood occurs in progressive infections blood cultures may be positive in advanced infections and in untreated cases are invariably positive at death The cutaneous lesion is not painful but tenderness of the regional lymph nodes does occur A history of occupational exposure to the disease is of great assistance in the diagnosis particularly in areas such as the United States where the disease is uncommon

Diagnosis of respiratory or intestinal anthrax is a much more difficult problem because of the mild and nonspecific nature of the early symptoms and the fulminating course of the advanced disease The organism may be *detected in sputum by microscopic* examination of stained smears cultural diagnosis is seldom accomplished during the life of the patient Textbooks of veterinary pathology may be consulted for descriptions and diagnostic procedures applicable to the disease in *animals (Hutyra and Marek 1922* Sterne 1958) The Ascoli thermoprecipitin reaction is of value in postmortem diagnosis Infected tissue is ground in saline heated for 5 minutes filtered and the filtrate is layered over appropriate antianthrax serum In positive reactions a *ring of precipitate is formed* at the interface between the two fluids Measurement of the antibody response to infection has been of little value in diagnosis

THERAPY

Antisera prepared by hyperimmunization of animals with living cultures were used for many years in treatment of anthrax It seem

that such antisera alone or in combination with neoarsphenamine produced a considerable reduction in the mortality of cutaneous anthrax. With the advent of the sulfonamides a further reduction in mortality was obtained. These drugs in their turn have been superseded by the antibiotics and the mortality of promptly diagnosed and adequately treated cases of cutaneous anthrax is essentially zero. The anthrax bacillus is susceptible to penicillin, tetracyclines, erythromycin, achromycin and chloromycetin; all have been found to be satisfactory for management of cutaneous anthrax in man. According to Gold (1955) terramycin, achromycin and erythromycin are the drugs of choice. Different strains of the organism may differ somewhat in susceptibility to the antibiotics. Emergence of antibiotic resistance has not been a significant problem in therapy, although virulent antibiotic resistant strains may be obtained by selection in the laboratory. Antibiotics rapidly halt the extension of the disease and sterilize the tissues, but they do not reverse the toxic processes initiated by the infection. The primary lesion usually progresses to formation of the typical eschar despite early therapy. The death of infected guinea pigs despite doses of streptomycin sufficient to destroy all viable organisms has been discussed above. Little information is available regarding antibiotic therapy of respiratory anthrax in man. The early symptoms are mild and nonspecific, and the existence of acute disease is seldom recognized until terminal symptoms appear. Treatment has usually been initiated late in the disease, and the unfavorable results provide no basis for estimating the possible effectiveness of earlier therapy. Experimental respiratory infection in monkeys may be suppressed by administration of penicillin, but following discontinuance of the drug the animals died. If active immunization were carried out while the infection was suppressed by penicillin the animals survived (Henderson et al. 1956).

EPIDEMIOLOGY

Although anthrax is primarily a disease of cattle, sheep, horses and goats, virtually all animals show some susceptibility, and the disease has been detected in nearly every country. It is particularly common in the Mediterranean region, Africa and Asia. Epizootics with heavy loss of livestock occur at intervals in enzootic areas. In Iran during 1945, for example, 1 million of the 15 million sheep were reported to have died of anthrax (Delpy and Kaweh 1946).

In the United States scattered outbreaks are widespread, but the disease is not a continuing problem except in certain areas. During the period 1945-1954, 3,447 outbreaks were reported from 39 states with loss of 17,600 head of livestock (Stein and Van Ness 1955). The 3 important endemic areas are the Gulf coast region of Louisiana and Texas, a portion of eastern South Dakota and Nebraska, and an area in central California. Widespread outbreaks were recorded as early as 1835; presumably the disease was introduced during early settlement, and the spread to new areas appears to be continuing. Climatic conditions and the nature of the soil evidently determine the establishment of the disease in certain areas and the periodic occurrence of epizootics (Minett 1952, Van Ness and Stein 1956).

In animals the disease is transmitted primarily by ingestion of contaminated forage or the carcasses of infected animals. The discharges of infected animals may contaminate pastures; at death of the animal the blood teems with bacilli and the fresh carcass is capable of infecting animals that feed upon it. The bacilli in the unopened carcass are killed rapidly by putrefactive processes, but sporulation may occur rapidly if the bacilli are exposed to air. Spores may be disseminated over wide areas by surface water, animals, birds and insects. Contaminated foods are frequently responsible for introduction of anthrax into new areas; imported bone meal was the cause of the extensive outbreak in the midwestern United States in 1952 (Stein and Stoner 1953). It is not clear what importance should be ascribed to saprophytic growth of the organism in favoring persistence of the disease in certain areas. The spores may remain viable for many years in soil, and some authorities consider that the epidemiology of the disease can be accounted for without assuming a saprophytic existence for the organism. However *B. anthracis* grows readily on ordinary laboratory media, and its growth requirements are no more complex than typical saprophytic members of the genus (Proom and Knight 1955). There would appear to be no

of infection that is necessary for development of immunity This difficulty was overcome by the introduction of nonencapsulated spore vaccines by Sterne (1939) The nonencapsulated mutants have lost the ability to form the glutamyl polypeptide and are essentially avirulent They sporulate well, and vaccines are readily prepared on a large scale These vaccines produce smaller losses among vaccinated animals than Pasteurian vaccines, and a single preparation is suitable for all species of animals They were first introduced and used on a large scale in South Africa and are replacing the Pasteurian vaccines elsewhere None of the living spore vaccines is generally considered safe for immunization of man

For many years attempts have been made to develop effective nonliving antigens Until recently none of the antigens produced in vitro contained significant protective antigen and their immunizing effectiveness was negligible The work of Gladstone and subsequent investigators in developing conditions for elaboration of the protective antigen has been described above Alum precipitated preparations of protective antigen elaborated in vitro have been shown to produce effective immunity in guinea pigs rabbits monkeys sheep and cattle (Wright et al 1954 Schlingman et al 1956 Darlow et al 1956) The antigen has been used in man without serious local or systemic reactions clinical trial in occupationally exposed workers is in progress The preparations have not reached a stage of development at which they can replace spore vaccine for large scale immunization of cattle and other animals

DIAGNOSIS

Prompt and accurate diganosis of cutaneous anthrax in man usually presents no serious difficulty The characteristic lesion begins as a small red macule which enlarges and local edema of varying extent gradually develops in the surrounding tissue A vesicle filled with clear fluid occupies a central position and is soon followed by satellite vesicles The organism may be easily demonstrated in the early lesion by cultural methods and may frequently be detected in stained smears Fluid and scrapings from the base of a previously unopened vesicle should be used for preparation of slides and of cultures on nutrient or blood agar Microscopic examination of Gram or Giemsa stained films will frequently reveal the organism and permit a rapid tentative diagnosis The lesion gradually becomes necrotic, with evolution of the black eschar characteristic of anthrax At this stage demonstration of the organism is less certain, and secondary infection may complicate the bacteriologic diagnosis Occasionally, inoculation of mice or guinea pigs will allow isolation of the organism when direct cultural methods fail Antibiotic therapy usually causes rapid disappearance of the organism even though evolution of the local lesion continues Therefore specimens for bacteriologic study should be obtained before therapy is initiated

Extension to the regional lymph nodes and to the blood occurs in progressive infections blood cultures may be positive in advanced infections and in untreated cases are invariably positive at death The cutaneous lesion is not painful but tenderness of the regional lymph nodes does occur A history of occupational exposure to the disease is of great assistance in the diagnosis particularly in areas such as the United States where the disease is uncommon

Diagnosis of respiratory or intestinal anthrax is a much more difficult problem because of the mild and nonspecific nature of the early symptoms and the fulminating course of the advanced disease The organism may be detected in sputum by microscopic examination of stained smears cultural diagnosis is seldom accomplished during the life of the patient Textbooks of veterinary pathology may be consulted for descriptions and diagnostic procedures applicable to the disease in animals (Hutyra and Marek 1922 Sterne 1958) The Ascoli thermoprecipitin reaction is of value in postmortem diagnosis Infected tissue is ground in saline heated for 5 minutes filtered and the filtrate is layered over appropriate antianthrax serum In positive reactions a ring of precipitate is formed at the interface between the two fluids Measurement of the antibody response to infection has been of little value in diagnosis

THERAPY

Antisera prepared by hyperimmunization of animals with living cultures were used for many years in treatment of anthrax It seems

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reason why the organism should not grow saprophytically under favorable conditions However, antibiotics elaborated by other *Bacilli* may limit proliferation in soil (McCloy, 1951)

In the United States approximately 50 cases of human cutaneous anthrax are reported annually additional unreported cases also occur The incidence is considerably greater in many countries and it has been estimated that from 20 000 to 100 000 human cases occur annually in the world (Glassman 1958) Man is infected by contact with infected animals (agricultural anthrax) or contaminated animal products (industrial anthrax) Agricultural anthrax occurs in farmers veterinarians and slaughterhouse workers Industrial anthrax occurs primarily in persons whose work brings them in contact with contaminated hair, wool, or hides although other products have been responsible for scattered outbreaks Dock workers are occasionally infected while handling contaminated hair and hides Extensive outbreaks of intestinal anthrax are reported occasionally from primitive areas in which meat from animals that have died of the disease is used as food and it is probable that such outbreaks are not uncommon

CONTROL MEASURES

Major emphasis in the control of human anthrax is placed on minimizing contact of man with infected animals or contaminated animal products The world wide problem is complex and the most appropriate measures vary in different areas and with different groups Economic and sociologic factors are interwoven with the purely medical and veterinary aspects of the problem Obviously the most satisfactory measure would be the elimination of the disease in animals Modern methods of immunization are capable of producing a marked reduction in incidence The organisms have been shown to remain viable in soil for many years and many agricultural areas are heavily contaminated The extent to which the disease can be eliminated by an effective program of immunization will doubtless vary with soil and climatic conditions Mass immunization has virtually eliminated the disease in South Africa (Sterne 1958) In colder countries in which the disease is sporadic outbreaks of animal anthrax have frequently been traced to imported food tuffs particularly bone meal This material may be rendered safe by proper steam treatment during processing

When outbreaks of animal anthrax occur prompt diagnosis, isolation of sick animals and suitable disposal of the carcasses are essential to limit the spread of the disease Prompt vaccination of healthy animals on the premises may also be indicated (Stein 1947) It is axiomatic that when anthrax is suspected autopsies should not be performed in the field so that further contamination of the area and sporulation of the organisms in the carcass may be avoided Disposal of carcasses should consist of complete cremation or deep burial Proper disinfection of the surroundings represents a difficult problem since most of the disinfectants that are effective with vegetative organisms are without action on anthrax spores Strong hypochlorite solutions are the reagents of choice and the period of exposure should be as long as is practical

Measures for the control of industrial anthrax include disinfection of animal products such as hides and hair that originate in areas in which the disease is widespread These materials may be heavily contaminated and economically feasible methods of disinfection that may be applied routinely without damage to the material are not easy to devise Observations on sterilization by heat have been reviewed by Schneiter and Kolb (1948) Washing with soap and exposure to warm formaldehyde are effective with hair and wool Measures to minimize the contact of workers with the contaminated materials are also of major importance Recent advances in immunization with nonviable preparations give promise that immunization of occupationally exposed workers may become practical in the future

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14

The Clostridia

Organisms of the genus *Clostridium* are anaerobic or micro aerophilic gram positive rods producing endospores which in many species are wider than the rods from which they arise A number of species decompose proteins or ferment carbohydrates Several are pathogenic and a number produce toxins *Clostridia* of one species or another are always present in soils and in the intestinal tracts of animals and man The majority are saprophytic and actively decompose organic matter in the soil Some of these (*C acetobutylicum*) are used in industry for the production of chemicals such as acetone and butanol

The pathogenic *Clostridia* include (1) the virtually noninvasive saprophyte *C botulinum* which is dangerous because of the potent toxin it produces in foods (2) *C tetani* which has slight invasive powers but produces so potent a toxin that even slight lodgement in the host is dangerous (3) frankly invasive *Clostridia* which cause gas gangrene and enterotoxemias in man and animals

MORPHOLOGY

All species consist of relatively large gram positive rods Some (e g *C tetani*) are easily decolorized and retain the stain only when young The pathogenic *Clostridia* vary in size from the large rods of *C novyi* Type B ($1.0 - 1.8 \times 8 - 12\ \mu$) to the more slender *C tetani* ($0.3 - 0.8 \times 4 - 8\ \mu$) Cultures may show a great diversity of shapes and pleomorphic forms including filaments or gonits and citrons are characteristic of species such as *C septicum* and *C chauvoei* All *Clostridia* form spores which are usually of greater diameter than the rods from which they arise The shapes and the positions of the spores vary considerably from species to species and these differences are of great assistance in identification and classification Prevot (1948) goes so far as to define orders on the basis of spore position Some species form spores freely in the media usually available while others rarely sporulate This is due to variations in reaction to the nutritional environment rather than to inherent differences in ability to sporulate because media can be devised in which even *C perfringens* will produce dense crops of spores Most *Clostridia* possess peritrichous flagellae and are actively motile However *C perfringens* is consistently nonflagellated and nonmotile A few species (e g *C perfringens*) form capsules

Bacteriologists familiar with aerobic organisms will be struck by the confusing pleomorphism of the *Clostridia* Not only may a variety of forms appear in a particular culture but also their frequency may vary from culture to culture from medium to medium and from strain to strain of the same species How

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exacting species such as *C hemolyticum* to make certain that they will support the growth of any pathogenic *Clostridium* which might be encountered

ISOLATION AND IDENTIFICATION

As the constitution of media available in different laboratories varies widely the list given here should be considered as indicating broadly the type of reagents needed Most are procurable commercially or can be made up from commercially available ingredients

Chopped Meat Medium This consists of infusion broth to which have been added meat particles prepared by extracting lean minced heart with water The infusion broth is filled into tubes to a depth of about 2 inches and about one third by volume of the meat particles is added The pH is adjusted so that it will be about 7 4 after autoclaving Glucose reducing agents growth factors etc may be added if required

Fermentation Reactions To 2 per cent proteose peptone and 0 5 per cent NaCl add agar and sodium thioglycollate to 0 1 per cent adjust pH and autoclave Before inoculation add sugars sterilized by filtration As heat sterilized sodium thioglycollate may be inhibitory it is often advisable to add this before inoculation as a filtered solution The indicator (brom thymol blue) is added when the tests are read

Production of Indol The fermentation medium plus 0 2 per cent sodium phosphate and 0 1 per cent dextrose can be used It can also be used for the vanillin violet reaction particularly if extra tryptophane is supplied

Reduction of Nitrate The fermentation medium with 0 1 per cent potassium nitrate

Liquefaction of Gelatin The fermentation medium plus 5 per cent gelatin

Protein Digestion Add 5 to 8 per cent fresh egg white or 10 per cent serum to infusion broth Mix in a blender dispense in tubes after the foam has subsided Autoclave

Milk Add 5 per cent skim milk to infusion broth adjust pH to 6 8 dispense in deep tubes with reduced iron or iron strips Autoclave

Blood Agar Plates Add 5 per cent fresh defibrinated blood to a suitable agar base The species of blood added has a significant effect on certain hemolytic reactions Double strength agar may be used for preparing stiff antiswarming plates

Egg Plates for Lecithinase Action Collect the yolk of an egg aseptically and mix with an equal volume of physiologic saline Add to



Fig 34 Colonies of *Clostridium* 48-hour growth on blood agar in an anaerobic jar *(Top left) C perfringens (top right) C novyi (bottom left) C histolyticum (bottom right) C sporogenes*

a suitable agar base in the proportion of 1 to 10

Specific Antitoxic Sera It is virtually impossible to characterize *Clostridia* completely without using antisera The minimum requirements are antisera against *C perfringens* Type A *C novyi* Types A or B *C septicum C tetani* and *C botulinum* (all types) If possible antisera against *C perfringens* β ϵ and ι toxins *C novyi* β and γ lecithinase and *C histolyticum* should also be obtained See also Reed and Orr (1943) Wetzler et al (1956)

Isolation

The decision as to whether or not a particular *Clostridium* was the cause of a particular condition is ultimately a clinical one However the bacteriologist should attempt to assess the number and the significance of the organisms present in order to provide the clinician with information that will help his decision A bald report that a number of specified *Clostridia* were present is of limited value For this reason there are disadvantages in transporting clinical material in the form of inoculated bottles of holding medium (e g chopped meat) since the relative numbers of

ever, variations in form in a culture should not be dismissed too lightly as manifestations of pleomorphism. Often it has happened that cultures submitted for typing or identification because of some unusual features could be resolved into mixtures of two or even more, species or types.

CULTIVATION

Clostridia are by definition anaerobic or micro aerophilic. The extent to which free oxygen inhibits growth varies with the species. *C. tetani* will not form surface colonies if the air pressure exceeds 5 to 15 mm of Hg, while *C. perfringens* grows readily at 150 mm and slowly at 350 mm (M'Leod 1930). *C. histolyticum*, *C. tertium* and *C. carnis* are able to produce visible colonies on *aerobic* blood agar plates.

The reasons why *Clostridia* are unable to tolerate appreciable amounts of free oxygen are not clear. It has been suggested that the metabolic activities of this group require the reducing conditions of a lowered oxidation reduction potential (Eh) which can be achieved only if the concentration of free oxygen is reduced. It is also possible that the deficiency of *Clostridia* in peroxidases and catalase may lead to the accumulation of lethal amounts of peroxide in the presence of free oxygen (M'Leod and Gordon 1925). Although no definitive evidence exists for the dependence of peroxide production on oxygen concentration, it may be significant that *C. novyi*, which forms considerable amounts of peroxide on exposure to air, is very sensitive to oxygen, while *Clostridia* which are able to grow aerobically have been said to produce slight amounts of catalase.

When the Eh of a liquid medium has been reduced to the level where growth can start the growing organism will keep its milieu reduced and multiplication will occur readily provided that the medium is adequate in other respects. The reducing conditions necessary are easily obtained in liquid media if the exposed surface is small in relation to the volume, and if the medium is de aerated immediately before inoculation by boiling for about 10 minutes. More exacting anaerobes may require the addition of a reducing sugar (glucose 0.2 to 1%), or reduced iron. More efficient reductants such as cysteine (0.01 to 0.1%), sodium thioglycollate (0.01 to 0.1%) or sodium formaldehyde sulfoxylate (0.01 to 0.05%) may be used to provide suitably reduced conditions for the most fastidious *Clostridia*. However, it must be remembered that the higher concentrations of the thiol containing reductants especially if heat sterilized are inhibitory to some strains. Any of the pathogenic *Clostridia* will grow in nutritionally adequate media to which chopped meat or brain have been added. The reducing systems involved are discussed by Lepper and Martin (1929).

For studying colonial morphology or for purifying mixed cultures, growth on or in solid medium is necessary. Separate colonies of any *Clostridium* can be obtained in deep agar shake cultures or in Veillon tubes. However, such cultures may be difficult to purify and for this and other reasons growth on surface plates is usually preferable even though conditions suitable for fastidious strains are more difficult to obtain. A number of methods have been described for obtaining anaerobiosis for surface growth. The simplest, most efficient and most widely used is incubation in a MacIntosh and Fildes anaerobic jar or its modification, the Brewer jar. In these the air is displaced by hydrogen and any residual oxygen is removed by combination with the hydrogen. This reaction is catalysed by palladinized or platinized asbestos contained in an electrically heated gauze cage within the jar. Palladinized alumina catalysts ("Deoxo") have now become available which are sufficiently active in the cold to obviate the need for a heating coil. Jars* fitted with cold catalysts are simpler to use and safer than the conventional jars. The growth of many *Clostridia* is improved by the addition of about 5 per cent CO_2 to the hydrogen.

All the *Clostridia* described as being pathogenic to man will grow on the surface of blood agar plates incubated in an anaerobic jar (see Fig. 34). Some *Clostridia* such as *C. novyi* Type B and *C. hemolyticum* (which are mainly of veterinary interest) are more exacting than those usually considered pathogenic for man and may require special media to ensure surface growth. It may help to pour a thin layer of agar over the surface of an inoculated plate or to add peptones containing high molecular weight polypeptides. Fresh batches of medium should be tested with an

* Obtainable from Baird and Tatlock, London.

organisms found in these cultures may differ significantly from those of the original material

Material submitted for examination will usually be from patients (e g exudates tissues) or articles such as clothing or dressings or suture material suspected of being sources of tetanus or gas gangrene and foodstuffs suspected of causing botulism or other food poisoning The methods of isolation described below apply mainly to clinical material

1 Gram stained films should be made from different parts of the specimen in order to gain an impression of the distribution and the relative numbers of the organisms present In *Clostridial* myositis films and cultures should be made from the affected muscle rather than from exudate or edema as some highly toxigenic species may not be very apparent unless the actual infected focus in the muscle has been located and filmed

2 Plate directly onto blood stiff blood (antiswarming) and egg yolk agar plates and incubate anaerobically and aerobically for 48 hours The jar should not be opened at 24 hours since even a short exposure to air may inhibit further development of microcolonies of some organisms (e g *C novyi*) A separate jar should be put up for examining at 24 hours if this is considered essential

3 Inoculate several tubes of chopped meat medium Heat some at 80 C for 10 minutes incubate for 24 hours and plate from these cultures as in (2) These plates will not reflect the relative numbers of the different *Clostridia* present in the original material as the chopped meat medium will select the more aggressive species

Plates from (2) and (3) should be examined in detail with a colony microscope and films of putative *Clostridial* colonies should be stained Table 36 summarizes the colonial and the microscopic appearances and the reactions on blood and egg agar of the *Clostridia* most likely to be encountered It should be remembered that colonial morphology on stiff agar plates is usually atypical It may be possible to fish selected colonies directly into chopped meat medium but it is always advisable to restreak the selected colony onto a plate in case the one fished into meat broth is impure Several streakings may be necessary before purity is assured

4 Inoculation of laboratory animals—usually guinea pigs—with clinical material triturated with 1 per cent CaCl will assist considerably in detecting and isolating pathogenic *Clostridia* particularly when the postmortem appearances point to a particular infecting organism Specific antisera can be used to block one organism or another in a mixed infection The heart s blood and the local lesion of dying or recently dead experimental animals should be cultured as shown in (1) (2) and (3) It should not be assumed that a particular *Clostridium* is pathogenic because it is isolated in large numbers from the heart s blood For example a guinea pig inoculated with a mixture of *C novyi* and *C bifermentans* will die of a *C novyi* infection although the circulation will contain mainly *C bifermentans*

5 The examination of dressings soils etc is carried out in a way basically similar to that used for clinical material the chief differences being in the methods used for obtaining representative samples of the specimen for culturing and animal inoculation Foodstuffs should be examined for the presence of toxins as soon as possible since delay may result in destruction of toxin and the loss of a chance of demonstrating conclusively the role of the food

6 When pure cultures of *Clostridia* have been isolated a tentative recognition can be attempted from the results of reactions in various media (see Table 37) Final identification depends on the production and the identification of toxins and the demonstration of pathogenicity in laboratory animals It must be emphasized again that the presence of known pathogens in tissue or in food is presumptive but not conclusive evidence of clinical infection

DISEASES CAUSED BY CLOSTRIDIA

Except during wars *Clostridia* are considered as playing a relatively minor role as agents of disease in man They are found mainly in wound infections (gas gangrene and tetanus) in enterotoxemia (*C perfringens* Type F) and in food poisoning (*C perfringens* Type A and *C botulinum*) Most clostridial infections of man are complications of wounds in which vascular damage has favored the development of anoxic conditions suitable for anaerobic growth Outbreaks of food poisoning occur as a result of exceptionally

TABLE 36 COLONY FORM REACTIONS ON BLOOD AND EGG AGAR AND MORPHOLOGY OF PATHOGENIC AND RELATED SPECIES OF CLOSTRIDIUM

	COLONY FORM*	HEMOLYSIS	PRECIPITATE ON EGG AGAR†	RODS‡	SPORES§	PATHOGENICITY FOR GUINEA PIGS	REMARKS
C botulinum Types A & B	B	+	X	S to T	OE	+	
C botulinum Types C D E	B E	+		T	OE	+	Motile colonies of Type C occur
C tetani	FG	+	Z	S	STC	+	
Gas gangrene group							
a Pathogenic species							
C histolyticum	C	+	Z	S	OEC	+	Micro aerophilic
C novyi Type A	D	+	X	S to T	OEC	+	Motile colonies occur
C novyi Type B	D E	+	W	T	OEC	+	Motile colonies occur
C perfringens Type A	A	+	W	TC	OER	+	
C perfringens B C D E F	A	+	w	TC	OER	+	
C septicum	DG F	+	Z	S to T	OEV	+	
C sordellii	DG A	+	w	T	OE	+	Variety of *C bifermentans*
b Associated species							
C aerofetidum	A	—	Z	T	OER	—	
C bifermentans	DG A	+	w	T	OE	—	See *C sordellii*
C butyricum	A	—		T	OEC	—	
C fallax	B	+		S to TC	OER	+	Pathogenicity lost on subculture
C sporogenes	D	+	w	S to T	OE	—	
C tertium	C	+	Z	S	OTC	—	Micro aerophilic
c Rare species							
C capitovale	C	+	Z	S to T	OTC	—	
C carnis	B	+	Z	T	OTC	+	Micro aerophilic
C cochlearium	B	+	Z	T	OTC	—	
C difficile	B	—	Z	S to T	OE	+	
C sphenoides	C	+	Z	T	STCR	—	Sporing cells cuneate
C tetanomorphum	E	±	Z	S to T	STC	—	

* *Colony forms on blood agar*

A Large raised colonies smooth to slightly ridged with entire to undulate margins 2 to 4 mm in diameter

B Smaller raised colonies smooth to irregular with entire undulate or serrate margin 1 to 3 mm in diameter

C Minute colonies raised smooth to irregular with entire to irregular margins with short rhizoids 0 2 to 1 mm

D Large colonies raised very irregular with wide spreading coarse rhizoid 3 to 6 mm diameter

E As D but smaller with finer rhizoids 1 to 2 mm in diameter

F Irregular granular colonies with delicate spreading rhizoids to irregular spreading rhizoidlike structures without a definite central colony

G Tendency to swarm

† *Colony forms on egg yolk agar*

W Wide zone 6 to 10 mm of precipitation extending well beyond the colony No iridescence

w Narrow zone of precipitate (2 — 4 mm) beyond colony No iridescence

X Narrow zone 2 to 4 mm of precipitation beyond the colony Iridescence over colony and precipitation zone

Y Precipitation only under colony Iridescence over colony

Z No precipitation no iridescence

‡ Vegetative morphology S—slender rods T—thick rods C—capsulated

§ Spore morphology O—oval S—spherical E—eccentric T—terminal C—clostridial R—rare V—variable in size shape and position

NOTES 1 Colony and cell morphology depend largely on the media used

2 The presence of capsules is probably more general than is indicated in the Table

organisms found in these cultures may differ significantly from those of the original material

Material submitted for examination will usually be from patients (e.g exudates tissues) or articles such as clothing or dressings or suture material suspected of being sources of tetanus or gas gangrene and foodstuffs suspected of causing botulism or other food poisoning The methods of isolation described below apply mainly to clinical material

1 Gram stained films should be made from different parts of the specimen in order to gain an impression of the distribution and the relative numbers of the organisms present In *Clostridial* myositis films and cultures should be made from the affected muscle rather than from exudate or edema as some highly toxigenic species may not be very apparent unless the actual infected focus in the muscle has been located and filmed

2 Plate directly onto blood stiff blood (antiswarming) and egg yolk agar plates and incubate anaerobically and aerobically for 48 hours The jar should not be opened at 24 hours since even a short exposure to air may inhibit further development of microcolonies of some organisms (e g *C novyi*) A separate jar should be put up for examining at 24 hours if this is considered essential

3 Inoculate several tubes of chopped meat medium Heat some at 80 C for 10 minutes incubate for 24 hours and plate from these cultures as in (2) These plates will not reflect the relative numbers of the different *Clostridia* present in the original material as the chopped meat medium will select the more aggressive species

Plates from (2) and (3) should be examined in detail with a colony microscope and films of putative *Clostridial* colonies should be stained Table 36 summarizes the colonial and the microscopic appearances and the reactions on blood and egg agar of the *Clostridia* most likely to be encountered It should be remembered that colonial morphology on stiff agar plates is usually atypical It may be possible to fish selected colonies directly into chopped meat medium but it is always advisable to restreak the selected colony onto a plate in case the one fished into meat broth is impure Several streakings may be necessary before purity is assured

4 Inoculation of laboratory animals—usually guinea pigs—with clinical material triturated with 1 per cent CaCl will assist considerably in detecting and isolating pathogenic *Clostridia* particularly when the postmortem appearances point to a particular infecting organism Specific antisera can be used to block one organism or another in a mixed infection The heart s blood and the local lesion of dying or recently dead experimental animal should be cultured as shown in (1) (2) and (3) It should not be assumed that a particular *Clostridium* is pathogenic because it is isolated in large numbers from the heart s blood For example a guinea pig inoculated with a mixture of *C novyi* and *C bifermentans* will die of a *C novyi* infection although the circulation will contain mainly *C bifermentans*

5 The examination of dressings soil etc is carried out in a way basically similar to that used for clinical material the chief differences being in the methods used for obtaining representative samples of the specimen for culturing and animal inoculation Food stuffs should be examined for the presence of toxins as soon as possible since delay may result in destruction of toxin and the loss of a chance of demonstrating conclusively the role of the food

6 When pure cultures of *Clostridia* have been isolated a tentative recognition can be attempted from the results of reactions in various media (see Table 37) Final identification depends on the production and the identification of toxin and the demonstration of pathogenicity in laboratory animals It must be emphasized again that the presence of known pathogens in tissue or in food is presumptive but not conclusive evidence of clinical infection

DISEASES CAUSED BY CLOSTRIDIA

Except during wars *Clostridia* are considered as playing a relatively minor role as agents of disease in man They are found mainly in wound infections (gas gangrene and tetanus) in enterotoxemia (*C perfringens* Type F) and in food poisoning (*C perfringens* Type A and *C botulinum*) Most clostridial infections of man are complications of wounds in which vascular damage has favored the development of anoxic conditions suitable for anaerobic growth Outbreaks of food poisoning occur as a result of exceptionally

TABLE 37 BIOCHEMICAL REACTIONS OF PATHOGENIC AND RELATED SPECIES OF CLOSTRIDIUM

Species	Milk	Dextrose	Maltose	Lactose	Salicin	Sucrose	Gelatin Liquefaction	Nitrate Reduction	Indol	Vanillin Violet	
C aerofoetidum	ACGS	+	+	+	+	−	+	+	−	−	
C butyricum	ACGS	+	+	+	+	+	−	−	−	−	
C perfringens	ACGS	+	+	+	∓	+	+	+	−	−	
C botulinum (non ovolytic Types B C D E)	A	+	+	−	+	∓	+	−	−	−	Carbohydrates variable
C carnis	AG	+	+	+	+	+	−	−	−	−	Micro aerophilic
C capitovale	AC	+	−	−	−	−	+	−	−	−	
C fallax	ACG	+	+	∓	∓	∓	−	−	−	−	
C chauvoei	ACG	+	+	+	−	+	+	+	−	−	
C paraputrificum	ACG	+	+	+	+	+	−	±	−	−	
C septicum	ACG	+	+	+	+	−	+	+	−	−	
C sphenoides	ACG	+	+	+	+	±	−	±	∓	−	
C tertium	ACG	+	+	+	+	+	−	+	−	−	
C botulinum (ovolytic Types A B)	AD	+	+	−	±	−	+	−	−	+	Micro aerophilic Carbohydrates variable
C bifermentans	CD	+	+	−	∓	−	+	−	+	−	
C histolyticum	CD	−	−	−	−	−	+	−	−	−	
C novyi Type B	D	+	+	−	−	−	+	−	−	−	Micro aerophilic Fastidious
C sordellii	CD	+	+	−	∓	−	+	−	+	−	
C sporogenes	D	+	+	−	−	−	+	+	−	+	
C novyi Type A	CG	+	+	−	−	−	+	−	−	−	Nitrates rapidly reduced but no nitrites produced
C tetani	C	−	−	−	−	−	+	+	+	−	Nitrates rapidly reduced Nitrites absent
C cochlearium	−	−	−	−	−	−	−	−	−	−	
C difficile	−	+	−	−	+	−	−	−	−	−	
C tetanomorphum	−	+	+	−	−	−	∓	−	−	−	

NOTE A acid C clot D digestion G gas S stormy fermentation + positive − negative ± usually positive ∓ usually negative
No attempt has been made to indicate the intensity of the reactions

favorable conditions for certain *Clostridia* These outbreaks are self limiting and the affected persons do not act as foci of further infections This is in sharp contrast with the situation among domestic animals in which diseases caused by *Clostridia* are well defined clinical entities causing constant and serious losses unless combated by the conventional methods of disease control

A greater awareness of the existence of well defined ecologic groups of pathogenic *Clostridia* might result in the finding of more types causing specific clinical syndromes in man—such as enteritis necroticans due to *C perfringens* Type F and food poisoning due to heat resistant *C perfringens* Type A The recognition of *C perfringens* Type D in man (Gleeson White and Bullen, 1955 Kohn and Warrack 1955) and the detection of antibody to the ι toxin of *C perfringens* Type E in the sera of soldiers in Korea (Marshall and Anslow 1955) are further examples of a possible trend

The epidemiologic and epizootiologic characteristics of pathogenic *Clostridia* are correlated with the type of toxin they produce Because of this more attention has been paid to the study of toxins than to the study of the less obviously important antigens such as agglutinogens A number of workers (see Smith 1955) have shown that the various species of pathogenic *Clostridia* can be divided into types based on differences in somatic flagellar and capsular agglutinogens Further work on such antigens would extend our knowledge of intimate intraspecific differences between *Clostridia* and throw more light on interspecific group reactions

GAS GANGRENE

About 30 per cent of war wounds become contaminated with *Clostridia* If the contamination is confined to the surfaces of the injured parts little toxin is formed and the results are usually no more than a slight retarding of the rate of healing Anaerobic cellulitis —a spreading infection of the fascial planes—is rather more serious This results in a varying usually slight degree of toxemia but the infection has little tendency to invade healthy tissue and is as a rule self limiting and easily treated In 5 per cent or less of wounds contaminated with *Clostridia* true gas gangrene develops The organisms colonize injured muscle especially where damage to the blood supply has occurred The damaged anoxic muscle provides favorable conditions for multiplication and toxin production and a rapid invasion and destruction of neighboring healthy muscle follows Since the host reaction in the muscle is negligible this condition is more correctly described as a myonecrosis than a myositis The prognosis is serious and speedy and energetic treatment is essential It should be clearly understood that gas gangrene is not synonymous with clostridial wound infection so that detection of *Clostridia* in a wound is not diagnostic of gas gangrene and not necessarily prognostic of a serious outcome Table 38 (from Smith 1949) summarizes the findings of several investigators on the clostridial flora of gas gangrene

TABLE 38 CLOSTRIDIAL FLORA OF GAS GANGRENE

	PER CENT OF CASES			
	1943	1944	1944	1946 Smith
ORGANISM	MacLennan (146 cases)	MacLennan (17 cases)	Stock (25 cases)	George (110 cases)
C perfringens	56	83	80	39
C novyi	37	47	48	32
C septicum	19	24	4	
C histolyticum	6	6		
C tetani	13		8	4
C bifermentans	4	35	20	54
C sporogenes	37	50	72	54
C tertium	30	59	8	3
C multifermentans				5
C butyricum	13		4	3
C capitovale	5			3
C fallax	1		4	3
C cochlearium	9		4	2
C putrificum	19			2
C regulare				2
C sphenoides	3			2
C paraputrificum				1
C hastiforme	3			
C tetanomorphum	2			

TABLE 39 CHARACTERS OF THE ANTIGENS AND THEIR DISTRIBUTION IN THE *Cl perfringens* TYPES

		MAJOR LETHAL ANTIGENS				MINOR LETHAL AND NONLETHAL ANTIGENS								NON αθδ HEMOLYSIS	
		α	β	ε	ι	γ	δ	η	θ	κ	λ	μ	ν		
TYPE	OCCURRENCE	Lethal necrotizing Ca dependent lecithinase	Lethal necrotizing	Lethal necrotizing	Lethal necrotizing	Lethal	Lethal hemolytic	Lethal	Hemolytic oxygen labile lethal	Collagenase	Proteinase	Hyaluronidase	Deoxyribo nuclease	Ox	Horse
A Classic	Gas gangrene (man) Intestinal commensal (man and animals) Putrefactive processes Soil etc	+++	–	–	–	–	–	(+)	++–	++–	–	+–	–++	(+)	(+)
Food poisoning	Patients and food in outbreaks of food poisoning	+++	–	–	–	–	–	–	(+)	++–	–	+–	++	–	–
B Classic	Lamb dysentery Foals	+++	+++	++–	–	++	(+)*	–	++	–	+++	+++	++	–	+–†
Iranian	Hemorrhagic enteritis of goats and sheep Iran	+++	+++	+++	–	?	–	–	+++	+++	–	–	+–	–	–
C Classic	Struck in sheep	+++	+++	–	–	++	+++	–	+++	+++	–	–	+–	–	–
Colorado	Neonatal hemorrhagic enteritis in calves and lambs	+++	+++	–	–	?	–	–	+++	+++	–	–	+–	–	–
Piglet	Enteritis in piglets	+++	+++	–	–	?	–	–	+++	+++	–	(+)	++	+++	+++
D	Enterotoxemia in adult sheep lambs goats and bovines Intestine of man	+++	–	+++	–	–	–	–	++	++	++	++–	++–	(+)†	+–†
E	Sheep cattle (? pathogenic)	+++	–	–	+++	–	–	–	++	++	++	(+)	++–	+–†	+–†
F	Enteritis necroticans (man)	+++	++–	–	–	++	–	–	–	–	–	–	+++	++†	++†

+++ = Produced by all strains ++ = Produced by most strains ++– +– = Produced by some strains (+) = Produced by very few strains Heavy type denotes production of large amounts of antigen

* = Antibody present in antisera of hyperimmune horses † = Hemolysis is produced only in the presence of antisera

(Brooks M E Sterne M and Warrack G H 1957 A reassessment of the criteria used for type differentiation of *Clostridium perfringens* J Path Bact 74 185 195)

C perfringens, *C novyi* and *C septicum* are recognized as the organisms mainly concerned in provoking gas gangrene There are almost certainly in these species intraspecific differences that influence or determine invasiveness and other qualities pertinent to pathogenicity Characteristic toxins and enzymes are produced which play roles of varying significance in invasiveness pathogenicity and virulence

The relation of other *Clostridia* to gas gangrene is somewhat doubtful The toxigenic *C histolyticum* has been found very occasionally in gas gangrene usually in association with other *Clostridia* *C sordellii* (also a toxin producer) has been found as the sole cause of fatal postoperative infections *C sporogenes* and *C bifermentans* are found in the majority of anaerobic wound infections Neither is pathogenic for laboratory animals in pure culture except in so far as occasional strains of *C bifermentans* will markedly digest tissues around the inoculation site and cause lesions resembling those produced by *C histolyticum* but not accompanied by toxemia or followed by death Other *Clostridia* found occasionally in wounds are not considered to be of much significance with the exception of *C tetani* which will be discussed separately See Smith and George (1946) Smith (1949 1955) Oakley (1954)

Clostridium Perfringens

This is the species most frequently associated with anaerobic cellulitis and gas gangrene in man It is divided into Types A B C D and F according to the main lethal toxin produced while Type F (the cause of enteritis necroticans in man) is separated on account of its heat resistance morphologic peculiarities and differences in minor antigens The main lethal toxins of the various *C perfringens* Types are shown in Table 38 A tabulation of the major and the minor toxins (based largely on Oakley and Warrack 1953) and of the ecologic relationships of types and subtypes of *C perfringens* is given by Brooks et al (1957) Up to the present only Type A has been found in anaerobic wound infection of man This type is probably not homogeneous and there is little doubt that it will be possible one day to distinguish subgroups or varieties of epidemiologic significance

Toxins and Enzymes

α TOXIN A culture of *C perfringens* Type A grown under optimal conditions for toxin production may contain up to 600 mouse LD_{50} per ml (Adams and Hendee 1947) This toxicity is due mainly to the lethal hemolytic and necrotizing α toxin although several other toxins and enzymes notably θ κ and μ toxins may also be present (see Table 39) The α toxin is the most important (pathologically considered) of the toxins of *C perfringens* Type A and is one of the few bacterial toxins of which the mode of action can be described in chemical terms It is an enzyme (lecithinase C) which catalyzes the hydrolysis of phosphate bonds with the liberation of phosphorylcholine from the phosphatides lecithin and sphingomyelin (Macfarlane and Knight 1941) It requires calcium for its activity and is therefore inactive in the presence of ions such as phosphate citrate and fluoride which sequester this metal The lecithinase activity of the α toxin is responsible for the opalescence and the turbidity produced when filtrates of *C perfringens* cultures (all types) are incubated together with a solution of egg yolk or when cultures are grown on the surface of egg yolk agar This opalescence is presumably due to the breakdown of the egg yolk lipoproteins lecithovitellin and lecithovitellinin Lecithinases are also produced to a lesser extent by other *Clostridia* e g *C novyi* *C hemolyticum* *C bifermentans* and by certain *Bacilli* e g *B cereus* All lecithinases except that produced by *C bifermentans* are immunologically distinct from the α toxin of *C perfringens* Since lecithin is widespread throughout the body the opportunities for attack by the toxin are numerous The hemolysis of red cells is doubtless due to the breakdown of lipoproteins but other factors must also be concerned since the red cells of different species are not equally susceptible to hemolysis although the phospholipids isolated from these cells are equally susceptible to hydrolysis (Macfarlane 1950) Other evidence which shows that the ability to attack lecithin is not the only factor determining toxicity is the fact that the lecithinases of *C bifermentans* show considerably less toxicity per unit of enzyme activity than the immuno

logically related *C perfringens* lecithinase (Miles and Miles 1950)

The action of the lecithinase in tissues results not only in generalized necrosis but also specifically, in the inactivation of enzyme systems that are dependent on lecithin in one way or another These include the magnesium activated adenosinetriphosphatase of muscle, which has a lecithin prosthetic group (Kielley and Meyerhof 1950) and the succinic dehydrogenase system which requires intact lecithin particles possibly for reasons of spatial configuration (Macfarlane and Datta 1954)

The best partially purified preparations contain about 10 000 mouse LD_{50} per mg (van Heyningen and Bidwell 1948 Roth and Pillemer 1953)

θ TOXIN The θ toxin is lethal hemolytic necrotizing and cardiotoxic and is produced by all types of *C perfringens* except Type F and the food poisoning varieties of Type A It is inactivated by mild oxidizing conditions such as standing in air and is reactivated by reducing agents such as cysteine and thioglycollic acid It is inactivated by cholesterol and its production is inhibited in media containing fat so that little or none will be found in cultures grown in chopped meat media Thus θ toxin resembles the oxygen labile cardiotoxic hemolysins of *C tetani Streptococcus pyogenes Diplococcus pneumoniae* and possibly also the δ toxin of *C novyi* Moreover it is related to these hemolysins immunologically since antibody to any one of them will neutralize any other The relatively high anti θ titers of many normal horse sera are probably the result of early exposure of these animals to one or more producers of O labile hemolysins The toxin has been purified by Roth and Pillemer (1955)

κ TOXIN This is a proteolytic enzyme which appears specifically to attack collagen (and its breakdown product, gelatin) and no other protein Such extreme specificity in proteolytic enzymes is perhaps unique A partially purified preparation of this enzyme made by Bidwell and van Heyningen (1948) contained 500 mouse LD_{50} per mg The toxin can be distinguished readily from the other active products of *C perfringens* by immunologic technics (Oakley et al 1946)

μ TOXIN This is an enzyme a hyaluronidase which hydrolyses hyaluronic acid the intercellular cementing ground substance of tissue As a result of its action on the intercellular cement, the toxin spreads rapidly through the skin and substances injected together with it will also be disseminated rapidly Although referred to as μ toxin this enzyme is much less toxic than κ toxin In addition to the lecithinase the collagenase and the hyaluronidase *C perfringens* Type A culture filtrates contain a fibrinolysin (which may be a protease) a desoxyribonuclease (η toxin), an enzyme which destroys the influenza virus receptor on red blood cells (a neuraminidase?) and a small amount of an enzyme which inactivates the blood group A substance

Clostridium Novyi

C novyi is divided into 4 types Table 40 modified from Oakley et al (1947) and Oakley (1955) summarizes our present knowledge of these Types A and B are distinguished by differences in the lecithinases which they produce (see Table 40) although the factor probably responsible for toxemia and death (α toxin) is the same in both Type A is commonly isolated from human gas gangrene

TABLE 40 TOXINS AND ENZYMES OF THE DIFFERENT TYPES OF *Clostridium novyi*

		Toxins and Enzymes					
Type	Distribution	Lethal necrotizing α	Lecithinase hemolytic necrotizing Lethal β	Lecithinase hemolytic necrotizing γ	O labile hemolysin δ	Indescence ε	Hemolysin ζ
A	Human gas gangrene	+++	−	+	+	+	?−
B	Black disease (necrotic hepatitis sheep)	+++	+	−	−	−	+
C	Osteomyelitis of buffalo	−	−	?+	−	−	−
D	Hemoglobinurea of cattle	−	+++	−			

while B is usually regarded as an animal pathogen However the latter is more difficult to cultivate than A and could be overlooked more easily Smith and Claus (1957) found that all proteolytic strains of *C novyi* which they examined were Type B so that this type may have been isolated from man more frequently than is generally thought In animals *C novyi* causes well defined diseases such as necrotic hepatitis of sheep (Type B) osteomyelitis of buffalo (Type C) and hemoglobinurea of cattle (Type D)

Toxins and Enzymes Cultures of *C novyi* Types A or B grown under optimal conditions for toxin production may contain 100 000 mouse LD_{50} per ml This toxicity is almost entirely due to the lethal and necrotizing α toxin about which little further is known The other toxins produced by Types A and B make only a small contribution to the total toxicity They are the γ and β lecithinases (which distinguish Types A and B immunologically) the δ and the ζ hemolysins produced only by Type A and ϵ toxin which is responsible for the iridescence shown by Type A strains on egg yolk agar (see Table 36) In the case of Type D (*C hemolyticum*) the β lecithinase is produced in very large amounts and becomes of overriding importance as the characteristic lethal toxin of this type The β and γ lecithinases are similar to the lecithinase of *C perfringens* differing from it and from each other immunologically and in the species of red cells which they attack Nothing further is known about the δ the ϵ and the ζ toxins Oakley et al (1947) should be consulted for a fuller discussion of the toxins of *C novyi*

CLOSTRIDIUM SEPTICUM

C septicum produces a potent α toxin which is necrotizing and lethal and probably hemolytic According to Bernheimer (1944) a single toxin is responsible for all three manifestations although other workers have not found the same close correspondence between hemolysis and the other activities This may mean that the hemolytic action of the toxin is dependent on activation or that more than one hemolysin is formed Further work is necessary to resolve existing contradictions This organism also produces a deoxyribonuclease (β toxin) described by Warrack et al (1951)

Only a single type of *C septicum* is recognized However toxin production and virulence for laboratory animals vary widely in different isolates and it is by no means unlikely that more intensive investigations would show the existence of epidemiologically important subdivisions It was shown by Guillaumie et al (1953) that horses hyperimmunized with *C septicum* filtrates produced antibody to *C histolyticum* α toxin as well as to *C septicum* α toxin and vice versa

CLOSTRIDIUM HISTOLYTICUM CLOSTRIDIUM SPOROGENES CLOSTRIDIUM BIFERMENTANS

C histolyticum although wide spread in nature is not often found in wound infections In MacLennan s (1943) series it was found in 10 of 143 cases but only once in pure culture All 10 cases ended fatally Hall (1945) considers that this organism is frequently overlooked in routine examinations and points out that it is more easily isolated on aerobic than on anaerobic media Smooth strains are pathogenic for laboratory animals The α toxin (Oakley and Warrack 1950) is lethal and necrotizing and the β toxin is a very active collagenase These are both distinct from the hemolysin

C sporogenes and *C bifermentans* are often found associated with other *Clostridia* in anaerobic wound infections Their role in the pathogenesis of gas gangrene is uncertain They cause no toxemia and it is doubtful whether they provoke gas gangrene although they may exacerbate infections by other *Clostridia* On occasion *C sporogenes* has been isolated as the only *Clostridium* in a wound infection However it grows so easily that it would be comparatively easy to overlook an admixture with a fastidious organism such as *C novyi* Sordelli (1922) isolated an organism closely resembling *C bifermentans* which caused fatal cases of postoperative gas gangrene Since then *C sordellii* has occasionally been isolated from fatal cases of gas gangrene in man and animals It is culturally and biochemically indistinguishable from *C bifermentans* except for its ability to produce a powerful toxin or toxins The lesions in experimental animals resemble those elicited by *C novyi* Although most workers consider that *C sordellii* is a variety of *C bifermentans* Prevot and Cordier (1941) regard them as

distinct species We feel that *C sordellii* should be considered as distinct from *C bifermentans* whether it is regarded as a type or a variety of the latter or as a species in its own right

PATHOGENESIS

Gas gangrene is a rapidly spreading myonecrosis following infection by pathogenic *Clostridia* of severely injured muscle particularly when the blood supply to the muscle has been interrupted The anoxia and the consequent anaerobic glycolysis result in a fall of the Eh to levels permitting multiplication and toxin production by the *Clostridia* A further result is an accumulation of lactic acid and a fall in pH which favors the activity of the catheptic enzymes in the muscle Amino acids thus become available for the *Clostridia* and enable them to grow at a higher Eh than would otherwise be possible (see Oakley 1954)

Once the anaerobes have gained a foothold in the damaged muscle they are aided by their extracellular toxins and enzymes in colonizing it and attacking undamaged muscle This is seen most clearly in the case of *C perfringens* Type A which has been studied most extensively The α toxin breaks down cells containing lecithin and interferes with their metabolism It renders capillaries more permeable to fluid and protein so that the resulting extravasation increases pressure within the muscle thus exacerbating the anoxia By breaking down cells it not only exposes them to attack but also provides more nutrient for the organism Since the production of θ toxin is inhibited in the presence of meat it is unlikely that this toxin plays an important part in the pathology of infection The κ toxin (collagenase) breaks down collagen barriers to the spread of the organism and the destruction of reticulin around capillaries may lead to further interference with the blood supply of the muscle The muscle is disintegrated by the destruction of its reticulin and collagen scaffolding and exposed to the action of catheptic enzymes and bacterial proteases The hyaluronidase (μ toxin) assists the spread of the organism by breaking down the interstitial ground substance and thus providing fermentable carbohydrate in the form of breakdown products of hyaluronic acid

It appears likely however that only the α toxin is essential for the initiation (at least) of the pathologic processes induced by *C perfringens* Evans (1943 1947) showed that guinea pigs in which gas gangrene had been produced by injecting *C perfringens* could be saved with α antitoxin, while θ, κ and μ antitoxins were ineffective alone and did not enhance the effect of α antitoxin when administered together with it It is still possible that the minor toxins may contribute to the development of the pathologic process once this has started Some workers have shown that the ability of different strains of *C perfringens* to produce α and other toxins in vitro may be correlated with their virulence others that it is not so (Evans 1945 Keppie and Robertson 1944) but it has not yet been shown that toxin production in vitro bears a close relation to toxin production during natural or induced infection

The ability of organisms to destroy tissues by means of extracellular enzymes does not necessarily connote a high degree of pathogenicity Some strains of the very proteolytic species *C sporogenes* and *C bifermentans* readily digest living tissue but the infections they cause on injection into experimental animals tends to be self limiting and unaccompanied by toxemia In wound infections the presence of these and other relatively nonpathogenic organisms alters the gross pathologic appearance of the lesion elicited by the frankly pathogenic *Clostridia* and may increase the severity of the infection

Infections with *C perfringens* have short incubation periods (9 to 48 hours) The muscle is soft and pulpy and there is little edema Fat droplets may be seen on the surface of the fluid exudate surrounding the affected muscle Erythrocytes are lysed and leukocytes are degenerated There is no putrefactive odor Experimental infection of the thigh muscles of guinea pigs causes a pink soggy appearance of the muscle On cutting into the lesion a pink stained fatty fluid exudes There is a moderate amount of gas and with some strains quite a marked digestion of the tissues The amount of edema varies considerably with the infecting strain There is no smell of putrefaction Infections with *C novyi* have longer incubation periods (about 5 days) and a higher mortality rate than those with *C perfringens* There is a very

marked edema and a profuse serous discharge from the wound (MacLennan 1943) There is little gas and little odor unless other proteolytic *Clostridia* are present In the experimentally infected guinea pig the abdominal wall and the subcutaneous tissues are infiltrated with a transparent colorless or pale pink edema which may be nearly a centimeter thick This edema surrounds the leg in which the injection was given The area immediately around the injection site may be hemorrhagic Small gas bubbles may be present and there is no putrefactive smell Organisms can be seen *readily near the site of injection but there are* relatively few in the extensive edema *C septicum* is not infrequently associated with gas gangrene in man and may be the only organism present The incubation period is 2 to 3 days There is little gas and considerably less serous exudate than with *C novyi* In guinea pigs death usually occurs within 24 hours The affected muscles are dark red sometimes almost black in color with some gas at the inoculation site and small bubbles of gas in the flank and along the abdominal wall A bloody edema may extend along the abdominal wall but is not nearly so prominent as that due to *C novyi* The liver is often pale and films made from it show large numbers of organisms Impression films made from the liver surface shortly after death show long chains and filaments of *Clostridia* The cadaver has a characteristic rancid smell that is almost diagnostic of *C septicum* or *C chauvoei* infection

While it is clear that the α toxin is largely responsible for the pathogenicity of *C perfringens* and that the gas gangrene induced by this organism is characterized by a profound toxemia it is by no means certain that this toxin is directly responsible for the toxemia even though many lethal doses of toxin may be produced in the infected muscle These doubts arise largely from the work of Macfarlane and MacLennan (1945) (see also Oakley 1954 van Heyningen 1955) They suggested that the toxemia and the anemia of gas gangrene might be due not to α toxin but to some toxic substance liberated by its action (or by some other action of *C perfringens*) on muscle They showed that *intravenous* injection of α toxin into rabbits led to intravascular hemolysis but that there was no intravascular hemolysis and no toxin detectable in the circulation after a lethal *intramuscular* injection Furthermore they were unable to recover α toxin from the muscle of injected animals or from gangrenous human muscle They suggested therefore that the toxin remained adsorbed to muscle *and that no significant* amount entered the circulation to cause systemic poisoning

It could be argued that if the toxin were released slowly into the circulation it would be undetectable and would be too dilute to cause intravascular hemolysis but would yet *be sufficient to allow for a lethal accumulation* at the susceptible sites in the body However, this concept is unlikely in view of the observation that patients could suffer from acute toxemia in spite of high concentrations of antitoxin in the blood That this might have been the result of an irreversible attachment of toxin to susceptible sites before antitoxin administration was improbable because surgical excision of the affected muscle resulted in a dramatic amelioration of the toxemia Therefore it would seem more likely that a toxemia producing substance passed from the affected muscle to the circulation but that this substance was not α toxin—since it was not neutralizable by specific antitoxin or detectable by any test for α toxin—but a substance produced by the action of α toxin The nature of this substance is not known but it may be relevant to mention the work of Kellaway and Trethewie (1941) who showed that perfusion of organs with filtrates of *C perfringens* types A B C and D led to a liberation of adenyl compounds which can bring about marked pharmacologic effects

Prophylaxis and Treatment

Potent antitoxins can be prepared by hyper immunization of horses with toxic filtrates of *C perfringens* *C novyi* *C septicum* and *C histolyticum* In experimental animals a high degree of protection can be achieved by a preceding inoculation with the specific antitoxin In man it is customary to give 9 000 units of *C perfringens* 4 500 of *C septicum* and 3 000 of *C novyi* α antitoxins prophylactically and about 3 times these amounts every 4 to 6 hours for therapy Treatment with antisera appears to be of benefit provided that and only provided that adequate surgery has

been carried out (see Macfarlane 1943, MacLennan and Macfarlane, 1944) Necrotic muscle must be removed as early and as radically as possible If all damaged muscle can be excised, the source of toxemia provoking substances and the bulk of the infecting organisms will be removed with it and the Eh of adequately vascularized muscle will be too high for the remaining *Clostridia* to multiply in it Moreover, early removal of devascularized muscle will prevent the fall in pH and consequent proteolysis which results in conditions suitable for clostridial multiplication (Oakley 1954)

There is little evidence of the usefulness of chemotherapy of gas gangrene of man At best it may supplement but not displace, surgical treatment The successful active immunization of humans against diphtheria and tetanus (see below) by means of appropriate toxoids naturally encouraged hopes of similar immunization of persons exposed to risk of gas gangrene Work on these lines was initiated by British and American workers during the World War II and it was found that an appreciable antibody response could be elicited in man and animals by the use of aluminum adsorbed formol toxoids (Altemeier et al 1952 Robertson and Keppie 1943 Barr et al 1945) There are no data on the effects of such active immunization on the subsequent exposure of man to gas gangrene However immunization of domestic animals with *C perfringens C septicum* and *C novyi* vaccines is of demonstrable value against diseases caused by these organisms

TETANUS

Tetanus is a toxemia due to infection of an injury (which may be quite insignificant) with *C tetani* It may also be associated with burns puerperal infections infections of the umbilical stump (tetanus neonatorum) and various surgical procedures in which the infection may originate from contaminated suture materials dressings plaster etc Although tetanus is usually regarded as typically a complication of war injuries the incidence in civil life is not negligible In Great Britain the mortalities in civil life distributed by age show two maxima the first during childhood and the second in later middle life

The incubation period in man is usually between 5 and 10 days, which allows for spore germination, growth and toxin production and development of evident symptoms of toxemia These are characterized by convulsive tonic contraction of voluntary muscles In man the first symptoms are generally muscular spasms in the region of the local infection followed by trismus which rapidly increases to fixation In some cases the symptoms remain localized near the site of infection

Clostridium Tetani

The tetanus bacillus is widely distributed in soils (20 to 47% of samples) and in animal feces (up to 30%) Therefore, wounds contaminated with soil may result in tetanus although this is by no means an invariable consequence of the presence of *C tetani* in a wound Tulloch (1919) for example isolated the organisms from 19 out of 100 wounds in which there was no evidence of tetanus On the other hand it has been reported that spores may be phagocytosed and remain dormant for several months and then initiate a fatal infection if they happen to be deposited in an injured area (contused wounds sites of injection of irritant substances See Smith 1955)

Young actively growing organisms are gram positive but most cultures are completely gram negative by 48 hours and very often by 24 hours The spores are terminal and round when completely ripe but usually appear slightly oval Surface colonies may be surrounded by an area of swarming and this habit can be utilized for the isolation of *C tetani* from mixed cultures (Fildes 1925) Deep colonies are semitransparent and woolly *C tetani* is not as easily grown on the surface as *C perfringens* but is less exacting than *C novyi* It can be divided into 10 agglutinating types on the basis of specific flagellar antigens All types produce a neurotoxin neutralized by one antitoxin

Toxin A culture of *C tetani* grown under optimal conditions for toxin production may contain up to 500 000 LD_{50} per ml but toxin production varies widely with the strain Although tetanus toxin was the classic example of an exotoxin it is now known that the young bacilli contain most of the toxin subsequently found in the medium (Raynaud 1947) Tetanus toxin was one of the first to be ob

tained in a highly purified crystalline state (Pillemer et al 1946) It is a simple protein with a molecular weight of 76 000 containing 10 000 000 mouse LD_{50} per mg Thus tetanus botulinum and dysentery toxins are the most poisonous substances known In the highly purified state the toxin rapidly and spontaneously loses its toxicity but not its antigenicity (i e toxoids) and at the same time its molecular weight appears to double (Pillemer et al 1949) In dilute solution the toxin whether crude or purified is highly susceptible to surface denaturation like many active proteins and should be protected by the addition of gelatin or broth to the diluent In contradistinction to this inactivation by dilution a number of workers have reported an increase of toxicity (potentiation activation) in solutions of the toxins in diluents containing broth serum organ extracts acetates citrates proteolytic enzymes (Traub et al 1946 Seki et al 1954) Potentiation appears to be demonstrable in some animals but not in others which suggests that the effect may not be on the toxin alone but on its interaction with the host However this work awaits confirmation since the control experiments designed to show that the potentiation was not merely a protection against denaturation do not appear to be conclusive

The toxin acts only on nerve tissue with which it unites and by which it can be inactivated in vitro (Wassermann Takaki reaction see Fulthorpe 1956) Its action is dependent on temperature for if a frog is inoculated with toxin and kept below 18 C no tetanus develops even though there may be a high concentration of toxin in the circulation However if the frog is warmed a few degrees it will die of tetanus (see Wright 1955) The primary biochemical lesion which the toxin causes in nerve tissue is not known but the pharmacologic consequences of this lesion have been studied extensively The main action of the toxin is on the neural elements of the central nervous system where it appears like strychnine to diminish or abolish synaptic inhibition (Brooks et al 1955 1957) To achieve this the toxin may like botulinum toxin (see below) prevent the release of the inhibitory transmitter substance or like strychnine become attached to the subsynaptic inhibitory areas on the motoneural membrane and prevent the inhibitory transmitter from acting upon it When injected into the anterior chamber of the rabbit s eye it acts on the cholinergic motor nerve of the *sphincter pupillae* of the iris and paralyzes the muscle (Ambache et al 1948) Botulinum toxin exerts a similar action (Ambache 1949) possibly by preventing the release of acetylcholine from the motor nerve endings However the actions of these two toxins differ for tetanus toxin is extremely lethal when injected into the medulla oblongata whereas botulinum toxin has no comparable effect (see Wright 1955)

In addition to the neurotoxin *C tetani* also produces small amounts of an oxygen labile hemolysin which like the other oxygen labile bacterial hemolysins is necrotizing and cardiotoxic

Pathogenesis

C tetani has limited powers of invasion which are not reinforced by the neurotoxin since this has no general histotoxic effect The hemolysin which is necrotizing may possibly play a supporting role Only a slight degree of local tissue destruction is needed for initiating infection and establishing the lowered Eh necessary for germination of the spores (Fildes 1929) Once multiplication has started toxin is formed rapidly and is so potent that quite small amounts will result in a fatal outcome The harmful effects of *C tetani* infections are entirely due to the neurotoxin

The mechanism by which toxin spreads from the site of injection to the susceptible sites in the central nervous system has been the subject of controversy for many years but it now seems certain that toxin travels first up the motor nerve trunks and then up the spinal cord This is essentially the route proposed originally by Meyer and Ransom (1903) Their views were opposed vigorously by Abel (see Wright 1955) who contended that the toxin was carried to the central nervous system exclusively in the blood stream More recent evidence supports the earlier workers For example different workers have shown in different ways (Friedemann et al 1941 D Antona 1951) that an intravenous dose of antitoxin which could protect against a given dose of toxin administered intravenously failed completely to protect against a

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BOTULISM

Botulism is not an infectious disease but a poisoning caused by eating food in which *C botulinum* has grown and produced toxin In man symptoms appear after periods varying from less than a day to several days after ingestion of the toxin depending on the amount taken They include vomiting constipation thirst double vision difficulty in swallowing and speaking and respiratory paralysis In survivors convalescence is slow and partial paralysis may persist for several months

A few cases of botulism due to wound infection have been reported (Davis et al 1951 for example) but for all practical purposes the organism may be considered as noninvasive Outbreaks of botulism among humans are sporadic and rare and would be regarded as insignificant were it not for the high fatality rate From 1899 to 1947 there were 462 outbreaks in the United States and Canada with 1 253 cases and 815 deaths In the United Kingdom only 4 outbreaks have been recorded In animals botulism occurs far more frequently than in man and may be so prevalent in some areas that husbandry becomes virtually impossible unless measures are taken to control the disease

Human cases result from the eating of smoked salted spiced or canned foods which have been allowed to stand for a period and eaten without being cooked In Europe most cases have been due to sausages (Latin *botulus* whence botulism) ham spiced meats game pastes potted meats and in Russia salt fish In the United States most cases have been due to canned especially home-canned vegetables and fruits such as olives string beans corn and peas Affected meats may appear obviously spoiled but the growth of *C botulinum* in canned vegetables may be slight and apparently insignificant although dangerous amounts of toxin may be formed Dack (1949) Tanner and Tanner (1953) should be consulted for further detail

Clostridium Botulinum

The natural habitat of the organism is the soil It is rather more prevalent in virgin soil than in cultivated manured or pasture lands In the United States it appears to be more common in the Rocky Mountain system and west of it It occurs less frequently in the states on the Atlantic seaboard and rarely in the Great Plains or the Mississippi River Valley (Meyer and Dubovsky 1922a) Type A (see below) is more common than Type B in the United States while the latter is more usual in Europe (Meyer and Dubovsky 1922b) The frequency with which *C botulinum* occurs in virgin soils and in vegetable matter of various kinds suggests that it is not as was once thought primarily an intestinal parasite It can be found in the feces of domestic animals in areas where botulism is enzootic but it has almost never been isolated from human feces

Botulinum organisms are divided into two species the strongly proteolytic (ovolytic) *C parabotulinum* and the less proteolytic (nonovolytic) *C botulinum* They are also divisible into Types A B C D and E according to the immunologic specificities of the toxins they produce There is a considerable diversity of cultural and biochemical behavior within these types so that it is possible to have representatives of both *C botulinum* and *C parabotulinum* in the same Types (e g in A and B) However the immunologic differences in the toxins are constant and clear-cut and since these characteristics are of definitive epidemiologic significance it is now customary to refer to all organisms producing botulinum toxin as *C botulinum* and to subdivide these into Types as recommended at the 6th International Congress of Microbiology (1953) Deep colonies of Types A and B may be round or lenticular while those of C and D resemble loose balls of cotton wool Types A and B are far more proteolytic than the virtually nonproteolytic C D and E Smith (1955) should be consulted for a more complete discussion of the cultural and biochemical characteristics of this species

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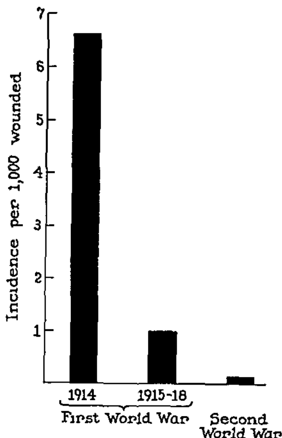


FIG 35 Incidence of tetanus in the British Army in two world wars Prophylactic passive immunization against the toxin was introduced toward the end of 1914 active immunization before the second world war (van Heyningen W E 1955 Reproduced with the permission of the Cambridge University Press)

similar dose administered intramuscularly However that same intramuscular dose of toxin failed to kill if the regional motor nerves to the injected muscle had been divided previously Thus it is clear that toxin injected into the muscles reaches the central nervous system by a route sheltered from circulating antitoxin in all probability via the motor nerves

A small dose of toxin injected into the hind limb of an animal is absorbed by the motor nerves and travels up the nerve trunk and results in ascending tetanus which affects first the muscles in the injected leg then those of the opposite leg then those of the back and the abdomen When a large dose of toxin is injected into a site from which direct absorption by motor nerves is not possible it first appears in the lymph from the area injected and thence passes into the blood stream from which it is absorbed by all the peripheral motor nerves This leads to descending tetanus, where the muscles first affected are those of the head and the neck followed by those of the arms the trunk and the legs

PROPHYLAXIS AND TREATMENT

Experience with troops during World War I showed clearly the value of prophylactic passive immunization with antitoxic horse serum Experience in World War II showed equally convincingly that tetanus could be eliminated by active immunization of troops with toxoid (see Fig 35) The incidence in World War II was only a tenth of that in the first 35 cases in the British army (15 in nonimmunized soldiers) and only 12 in the American army On the other hand tetanus was relatively frequent and accompanied by a high mortality in the German and the Japanese armies in which active immunization was not practiced

In civil practice injured persons are usually given prophylactic hyperimmune serum and this confers a high degree of resistance to tetanus for a limited time A complication is that spores of *C tetani* may germinate late in almost healed wounds by which time passive protection may have fallen below the safe limit A further difficulty is that persons who have had previous inoculations of serum may have become immune to it and will eliminate a subsequent injection of antitetanic serum so rapidly that it may prove to be ineffective The success of active immunization of soldiers suggests that it would be desirable to immunize civilians similarly In 1943 general active immunization of young children against both tetanus and diphtheria was made obligatory at Lyons and since then the practice has spread to other places in France and in North America

The results obtained in the treatment of tetanus with antiserum have been poor because it is not possible to dissociate the complex of toxin and the susceptible moiety of nervous tissue Nevertheless antiserum should be given to neutralize toxin which is being formed and has not yet been fixed Death following injury caused to nerve tissue by tetanus toxin is usually the result of respira

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Man is affected mainly by Type A less frequently by Types B and E and never (with

one possible exception) by C or D These differences almost certainly reflect variation in human susceptibility to the different types of toxin as much as in the degree of exposure to one or another of them For example, there is a much lower case mortality (20% or less) in Type B than in Type A (70%) intoxications and recovery from Type B has occurred even when appreciable amounts of toxin could be demonstrated in the blood In some areas (e g South Africa) where there is considerable exposure to C and D toxin no human cases of intoxication have occurred Only Types C and D have been found in herbivors in nature

The relatively high resistance of the spores of *C botulinum* to physical and chemical agencies set a problem for the canning industry where sterilization in bulk is necessary and where excess heating is deleterious to the product The safety record of commercial canned food is evidence that the problem has been solved (Smith 1955 Tanner and McCrea, 1923) Generally speaking spores may survive several hours at 100° C, and up to 10 minutes at 120° C The spores of Type E are much less resistant than those of the other types As with other *Clostridia* and with *Bacilli* great variation in resistance is found in different strains and in different cultures of the same strain

Toxins Cultures of *C botulinum* grown in the ordinary way under optimal conditions for toxin production may contain 2 000 000 mouse LD_{50} per ml and when grown in cellophane sacs suspended in culture medium yields as high as 200 000 000 LD_{50} per ml have been obtained (Sterne and Wentzel 1950) Toxin appears to be formed within the organisms and released on autolysis Production does not seem to depend on active multiplication since toxin is formed rapidly in resting cells (Kindler et al 1956)

Each Type of *C botulinum* is characterized by a toxin immunologically distinct from that of any other type While hyperimmune sera to Types A and B are monospecific hyperimmune sera to Type C usually contain a proportion of D antitoxin and vice versa This probably is due to Types C and D possessing a moiety of the heterologous toxins (Mason and Robinson 1935) Although the toxins are immunologically distinct their pharmacologic effects are identical and are effected through injury to the peripheral nervous system They act at the myoneural junction, apparently by preventing the release of acetylcholine from the demyelinated ends of the cholinergic motor nerves There is no effect on the peripheral adrenergic nerves It is not known how the release of acetylcholine is inhibited for there appears to be no direct influence on the enzymes synthesizing or breaking down acetylcholine (see Wright 1955 for a comprehensive and stimulating review of the pharmacology of botulinus and tetanus neurotoxins)

Although the different types of botulinum toxin have the same pharmacologic action they differ greatly in their relative pathogenicities for different animal species For example, the ratio of the lethal doses for mice and fowls are 1 15 for Type A 1 2,000 for Type C 1 100 000 for Type D and 1 25 for Type E Moreover, there is considerable variation in the ratio of oral to parenteral toxicity amon the different types and for different animals These differences are almost certainly of major epidemiologic significance

The botulinum toxins that have been purified all contained about 30 000 000 mouse LD_{50} per mg Type A was first purified and crystallized by Lamanna et al (1946) and Abrams et al (1946) and Type B was obtained in a highly purified but amorphous condition by Lamanna and Glassman (1947) while an improved procedure was described by Duff et al (1957) Type D toxin appears to be more lethal for mice than A or B, because *unpurified* filtrates of cultures grown in cellophane (Sterne and Wentzel 1950) showed a toxicity little less than that of crystalline A toxin A purified type D preparation (Wentzel et al 1950) showed potentiation in acid gelatin phosphate to about 20 000 times the toxicity of Type A toxin It would be most interesting to obtain confirmation of this remarkable but as yet isolated observation

The molecular structure of botulinum toxins appears to be unstable Purified Type A toxin was shown to be a protein of a molecular weight of about 1 000 000 (Putnam et al 1948) but Wagman and Bateman (1951 1953 Wagman 1954) have shown that under certain conditions the molecule may dissociate into fragments of molecular weight of approximately 70 000 some of which are inert and

others more toxic than the original molecule Types B and D also seem to occur in forms with different molecular weights (Lamanna and Glassman 1947 Wagman and Bateman 1951 Duff et al 1957 Wentzel et al 1950) Thus botulinum toxin molecules do not appear to be fixed entities but structures capable of dissociating and repolymerizing in various ways Such changes may account for differences in the behavior of various types and preparations and possibly also for the ability of the toxin to pass from the gut into the circulation

In addition to the neurotoxins *C botulinum* also produces small amounts of a hemagglutinin an O labile hemolysin and a hemolytic lecithinase (see Wright 1955)

Pathogenesis

The disease is entirely toxemic and can be exactly simulated by parenteral or oral administration of the isolated toxin although it must be remembered that purification separates toxin from factors promoting gut permeability (Coleman 1954) It is one of the few toxins and the only one of such formidable potency that is not destroyed and indeed may be activated by the acid conditions and by proteolytic enzymes in the gut (Boroff et al 1952 Duff et al 1956) It is unstable in the alkaline conditions prevailing in the greater part of the small intestine and although it can survive the action of proteolytic enzymes in vivo Halliwell (1954) observed that it could be inactivated by several proteolytic enzymes in vitro but that no proteolytic digestion took place during the inactivation The toxin can be absorbed through the respiratory mucous membranes (an additional hazard to laboratory personnel working with dried toxin) as well as the gut walls It is difficult to understand how so large a molecule can make this passage unless the disaggregation mentioned previously plays a part or unless the high molecular weights found experimentally are artefacts due to purification After absorption from the gut toxin can be found in the blood whence it presumably is absorbed by the peripheral nervous system

Prophylaxis and Treatment

Since the toxins of the different types of *C botulinum* are serologically distinct antisera that are used prophylactically should either be specific or be polyvalent to the extent of containing antibodies to A B and E the types to which man is naturally susceptible Antisera should be given to all persons suspected of having partaken of contaminated food Since the risk from botulism is ordinarily so slight there is no reason for active immunization with toxoid on a large scale although it is advisable to protect laboratory workers at special risk

Treatment with antitoxin has proved to be of little use in Type A intoxications probably because of the virtual irreversibility of the combination of toxin with nerve tissue and possibly because too little serum of too poor a quality was used It is more difficult to assess the value of serum in Type B intoxication in which recovery has occurred even after toxin has been demonstrated in the circulation It is rational to think that benefit would result from the neutralization of such circulating toxin Better antisera may conceivably improve prognosis As in tetanus the lesion caused by botulinum toxin may regress and the paralyses and other clinical manifestations of the disease gradually disappear For this reason artificial respiration should be maintained for long period by mechanical means if necessary (Legroux et al 1944 Mouquin et al 1944)

MISCELLANEOUS INFECTIONS

Clostridium Perfringens Type A

McClung (1945) and Hobbs et al (1953) isolated *C perfringens* Type A from cases of food poisoning and from food suspected of causing food poisoning in circumstances which strongly incriminated this organism It has since been found to be associated with a number of similar outbreaks in different parts of the world The characters of the strains isolated are such as to suggest that they form a well defined ecologic subdivision of Type A It is heat resistant produces little α toxin and no θ toxin

Clostridium Perfringens Type D

This organism which produces ϵ toxin and is the cause of pulpy kidney disease in sheep has been isolated from man twice recently (Gleeson White and Bullen 1955 Kohn and

Warrack 1955) Its significance in man is not known

Clostridium Perfringens Type E

Marshall and Anslow (1955) examined sera from a number of cases of epidemic hemorrhagic fever from Korea and found that several of these had antibody to the ι toxin of *C perfringens* (the main lethal toxin of Type E) It was not suggested that Type E stood in any etiologic relationship to epidemic hemorrhagic fever *C perfringens* Type F was not isolated from any of these cases so that the reason for the ability of these sera to neutralize ι toxin is as yet obscure

Clostridium Perfringens Type F

In 1949 Zeissler and Rassfeld Sternberg described an outbreak in Northern Germany in which a number of fatalities occurred of an enterotoxemic disease of man characterized by a sloughing necrotic enteritis This disease is analogous to the specific *Clostridial* enterotoxemias of animals and was shown to be caused by a *C perfringens* which produced large amounts of β toxin (Oakley 1949) Because the strain was markedly heat resistant and showed some morphologic peculiarities and because it lacked most of the *perfringens* minor toxins it was classified as a new type Type F rather than Type C in which its production of β toxin would naturally have placed it In view of the heterogeneity now known to exist in Type C it might be as well to transfer Type F to this group pending the results of further investigations (Brooks et al 1957)

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15

The Enteric Bacteria

INTRODUCTION

The enteric group of bacteria (*Enterobacteriaceae*) includes a large number of species of gram negative nonsporulating rods whose natural habitat in most instances is the gastro intestinal tract of man and other animals However this designation fails to acknowledge that other groups of organisms such as bacteroides enterococci and clostridia also are found in the gut and actually outnumber the so called enteric bacteria Some of the enteric bacteria are pathogenic for man and cause various types of gastro-intestinal diseases such as typhoid and other enteric fevers gastro-enteritis (*Salmonella*) or dysentery (*Shigella*) Others (e g the *Escherichia*) appear to lead a saprophytic existence in the intestinal tract but may cause pathologic processes in other parts of the body such as the genito urinary and the respiratory systems The *Aerobacter* group occurs most commonly in nature in soil and on grain though often it is isolated from the gut There are no simple differential criteria for these enteric organisms and classification is based on morphologic characteristics biochemical reactions antigenic properties and ecologic considerations Even when all these criteria are invoked some organisms fail to exhibit all the characteristics of a single group appearing to occupy an intermediate position between the main groupings

As a general rule the enteric bacilli grow readily on ordinary media They are aerobes or facultative anaerobes and characteristically they ferment a wide range of carbohydrates Many are actively motile and at least one group commonly possesses easily demonstrable capsules Their antigenic structure forms a complex mosaic which often results in serologic interrelationships between different genera and species

The enteric bacteria will be considered under the following groups

The coliform group is characterized by the prompt fermentation of lactose usually with the production of acid and gas *Escherichia coli* is a normal apparently harmless inhabitant of the intestinal canal but frequently causes infections of the urinary tract and other organs The closely related organism *Aerobacter aerogenes* is found most frequently in soil and on grain Certain other closely related bacilli are usually classified with these organisms The paracolon bacilli ferment lactose slowly frequently only after several days of incubation Their habitat is the intestinal tract of man and animals where for the most part they have no significance as causative agents of gastro intestinal disease *Klebsiella pneumoniae* is encapsulated and nonmotile and a saprophyte of the intestinal and upper respiratory tract but may cause inflammatory lesions in the lower respiratory tract and elsewhere

The Salmonella include the causative agents of typhoid fever paratyphoid and other enteric fevers gastro enteritis various types of septicemic infections and certain diseases of the lower animals as well Characteristically they fail to ferment lactose The Salmonella group is considered independently in Chapter 16

The Shigella include the causative agents of bacillary dysentery in man In contrast with those of other groups these organisms are nonmotile and with one important exception

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The Shigella include the causative agents of bacillary dysentery in man In contrast with those of other groups these organisms are nonmotile and with one important exception

Table 41 Biochemical Reactions of Enteric Bacteria

Organism	Motility	Fermentation Reactions: Glucose	Lactose	Sucrose	Mannitol	Salicin	Rhamnose	Dulcitol	Inositol	Sorbitol	Arabinose	Xylose	Indol	Hydrogen Sulfide	Gelatin Liquefaction	Russell's Double Sugar: Slant	Russell's Double Sugar: Butt	Citrate Utilization	Acetylmethylcarbinol (V P)	Methyl Red	Trimethylamine Oxide Reduction
E coli	+	AG	AG	V	AG	V	AG	V	—	AG	AG	AG	+	—	—	A	AG	—	—	+	
A aerogenes	V	AG	AG	AG	AG	AG	AG	V	AG	AG	AG	AG	—	—	—	A	AG	+	+	—	
Paracolon	V	AG	(V)													Alk	AG	V	V		
Kl pneumoniae	—	AG	AG	AG	AG	AG	AG	V	V	AG	AG	AG	—	—	—	A	AG	+	—	—	
S typhosa	+	A	—	—	A	—	—	—	—	A	—	V	—	+	—	Alk	A		—	+	
S paratyphi	+	AG	—	—	AG	—	AG	AG	—	AG	AG	—	—	—	—	Alk	AG		—	+	
S schottmuelleri	+	AG	—	—	AG	—	AG	AG	V	AG	AG	AG	—	+	—	Alk	AG		—	+	
S hirschfeldii	+	AG	—	—	AG	—		AG	—	AG	AG	AG	—	+	—	Alk	AG		—	+	
S typhimurium	+	AG	—	—	AG	—		AG	V	AG	AG	V	—	+	—	Alk	AG		—	+	
S choleraesuis	+	AG	—	—	AG	—	AG	V	—	AG	—	AG	—	V	—	Alk	AG		—	+	
S enteritidis	+	AG	—	—	AG	—		AG	—	AG	AG	AG	—	+	—	Alk	AG		—	+	
S gallinarum	—	A	—	—	A	—		A		—	A	A	—	V	—	Alk	A				
Sh dysenteriae Type 1	—	A	—†	—	—	—	—	—		—	—	—	—	—	—	Alk	A	—	—		—
Sh dysenteriae Type 2	—	A	—	—	—	—	V	—		V	V	—	+	—	—	Alk	A	—	—		—
Sh dysenteriae Types 3 7	—	A	—	—	—	—	V	(V)		V	A	V	V	—	—	Alk	A	—	—		—
Sh flexneri	—	A	—	V	A*	—	V	V		V	V	—	V	—	—	Alk	A	—	—		—
Sh boydii	—	A	—	—	A	—	—	V		(V)	A	(V)	V	—	—	Alk	A	—	—		—
Sh sonnei	—	A	(A)	(A)	A	—	A	—	—	—	A	V	—	—	—	Alk	A	—	—		+
Proteus vulgaris	+	AG	—	AG	—	AG	—	—	—	—	—	AG	V	+	+	Alk	AG	—	—	+	
Proteus morgani	+	AG	—	V	—	—	—	—	—	—	—	—	+	—	—	Alk	AG	—	—	+	
Ps aeruginosa	+	A											—	—	+			+	—	—	
Alcaligenes faecalis	+	—	—	—	—	—	—	—	—	—	—		—	—	—	Alk	—				

A = Acid AG = Acid and gas Alk = Alkaline + = Positive — = Negative V = Variable ( ) = Delayed

* = A few strains fail to ferment mannitol

† = A few strains are late lactose fermenters

fail to ferment lactose The Shigella group will be considered independently in Chapter 17

The Proteus group is comprised of organisms widely distributed in nature Although they are frequently found in the intestinal tract of man their causal relationship to enteric disease is with one exception doubtful Elsewhere in the body they may cause primary or secondary infections especially in the genito urinary tract They are actively motile decompose urea and fail to ferment lactose

For the sake of convenience *Pseudomonas aeruginosa* and *Alkaligenes fecalis* will be considered at the end of the present chapter

The biochemical reactions of the enteric bacteria are presented in Table 41

THE COLIFORM BACILLI

The coliform group includes the following gram negative bacilli *Escherichia coli* *Aerobacter aerogenes* the paracolon bacilli and *Klebsiella pneumoniae* The first two are often referred to as the colon aerogenes group *E coli* is a normal inhabitant of the intestinal tract of man and animals *A aerogenes* is found most frequently on grains and plants but also occurs in the feces of man and animals Because of its predominantly intestinal origin *E coli* is used as an indication of pollution of water with fecal material whereas the presence in water of other bacteria which like *A aerogenes* have other natural habitats does not necessarily indicate fecal pollution

Organisms of the colon aerogenes group cause infections in man which are primarily of a localized nature often involving the genito urinary tract or organs having an anatomic relationship to the intestinal tract e g gallbladder peritoneum and appendix

Escherichia Coli

E coli (synonym *Bacterium coli*) was isolated from feces by Escherich in 1885 It is found universally in the intestinal tract of man and animals and being the predominant organism in the colon is commonly referred to as the colon bacillus

E coli is a gram negative bacillus which commonly occurs as short rods from 2 to 3 μ long and about 0 6 μ in breadth and may form chains Occasionally very long filamentous forms are seen Most strains are motile *E coli* does not produce spores Some strains possess a definite capsule and growth at low temperatures favors the demonstration of this structure (Morgan and Beckwith, 1939) which may be identified by specific serologic reactions (e g capsular swelling)

E coli is facultatively anaerobic and grows on all ordinary laboratory media The optimum incubation temperature is about 37 C On beef extract agar it usually forms circular convex smooth colorless colonies with regular edges but some colonies which probably represent rough dissociants may have an irregular surface and edge By transmitted light the growth has a granular appearance On blood agar some strains produce hemolysis Growth in broth produces uniform turbidity with some sediment the cultures have a characteristic fetid odor

E coli ferments a variety of carbohydrates including dextrose lactose maltose mannitol and xylose but not dextrin or starch with the production of acid and gas Sucrose salicin and raffinose are attacked by some strains but not by others Colon bacilli form indol and do not liquefy gelatin H_2S is not produced

Colon bacilli are killed at a temperature of 60 C for 30 minutes They are more susceptible than the salmonella to the inhibitory action of such compounds as brilliant green dye and sodium desoxycholate The composition of certain differential media which are designed for the isolation of salmonella from the feces is based upon these differences in susceptibility

E coli is serologically heterogeneous and the antigenic pattern of the various species has been studied by Kauffmann (1951) who has established a diagnostic antigenic schema analogous to that available for the salmonella group he has divided them into O groups which are numbered 1 to 112 Besides the traditional O and H antigens capsular or envelope (K) antigens are recognized these are further subdivided into thermolabile (L and B) and thermostable (A) antigens The former are chiefly envelope antigens corresponding to the Vi antigens of salmonella In contrast the A antigens are usually of the visible capsular type Strains containing K antigens are more toxic and more resistant to the normal defense mechanisms of the host than are other strains Strains of *E coli* are classified on the basis of their O antigens and clinical evidence suggests that some of these O groups are more likely to be found in appendicitis

peritonitis and infections of the genito urinary tract than are other strains, suggesting a correlation between pathogenicity and antigenic structure Some strains of *E coli* have somatic antigens which are serologically identical with those occurring in certain members of the salmonella group

E coli undergoes dissociation to give rough and smooth colonial types The smooth round translucent colonies of the S form contrast with the irregular colonies of the R form which have a dull surface and opaque character Mucoid forms occur and appear more frequently when cultures are grown at low temperatures

Whereas *E coli* is a harmless and perhaps useful inhabitant of the intestines of man and animals under certain conditions it may assume the role of a pathogen, especially in the invasion of organs anatomically related to the intestinal tract such as the appendix gall bladder peritoneal cavity kidneys and bladder In appendicitis and peritonitis the colon bacillus commonly occurs in the tissues along with a variety of other organisms It is one of the most common invaders of the peritoneum following perforation of some part of the intestinal tract *E coli* appears to be the predominant organism in many cases of the suppurative form of cholecystitis Acute infections of the urinary tract including pyelitis pyelonephritis and cystitis are caused most frequently by *E coli* Along with the other organisms that may occur on the skin *E coli* is also found in wound infections but much less frequently than streptococci or staphylococci except when the wound has been contaminated with urine or feces Colon bacilli may gain access to the blood stream particularly in infants in the agonal stages of diseases and immediately after death Furthermore with the use of antibiotics such as penicillin which have their most pronounced action on gram positive organisms gram negative organisms such as *E coli* may become predominant in the upper respiratory tract and cause pneumonia

Recent investigations have demonstrated that certain strains of *Escherichia coli* cause infantile diarrhea and gastro enteritis Bray (1945) Giles and Sangster (1948), and Taylor Powell and Wright (1949) have independently isolated a serologically distinct strain of the colon bacillus (O111) from the feces of sick infants during the course of outbreaks of infantile diarrhea Subsequently Giles et al (1949) isolated another serologically distinct type (O55) from other outbreaks of the disease In all these instances the relationship between the presence of the specific organism and the occurrence of clinical disease was a close one while the incidence of positive cultures from the stools of healthy control babies was low Other investigators have reported a favorable therapeutic response to chloramphenicol and chlortetracycline which eliminated these strains of *E coli* from the gut thus providing ancillary indirect evidence for their etiologic role in enteritis in infants Subsequent studies have revealed that most infections were associated with two specific O antigenic groups, O55 and O111 in various parts of the world Furthermore Neter and Shumway (1950) produced diarrhea in an infant fed *E coli* (O111) and Ferguson et al (1952 1953) showed that volunteers fed *E coli* O111 or O55 developed gastro enteritis while those fed another strain of *E coli* did not

E coli is susceptible to the therapeutic action of the sulfonamides streptomycin chloramphenicol, and the tetracyclines, penicillin is relatively ineffective At present sulfonamides chlortetracycline and chloramphenicol appear to be the drugs of choice in the treatment of infections due to *E coli* These drugs may be used prophylactically to prevent infections due to contamination of the peritoneal cavity with fecal material as a result of injury or surgical operations

AEROBACTER AEROGENES*

A aerogenes (synonym *Bacterium aerogenes*) also described by Escherich, occurs less frequently in the intestinal tract than *E coli* and is found more often on grains and plants (Griffin and Stuart 1940) The difference in the usual habitat of these two closely related organisms has led to a detailed study of their specific differentiation because of the practical importance of the use of *E coli* as an indicator of the fecal pollution of water

* Kaffmann (1951) has suggested that *Aerobacter* be combined with *Klebsiella* in one genus bearing the latter name since they possess so many common characteristics and are not distinguishable antigenically

A aerogenes is often shorter than *E coli* and more commonly exhibits easily demonstrable capsules It is facultatively anaerobic and may be motile or nonmotile Growth on agar is often of a mucoid character with convex smooth colonies It grows more profusely at temperatures below 37 C with an optimum near 30° C Growth in broth produces a pellicle and a more viscous deposit

A aerogenes differs from *E coli* in producing less acid but more gas during fermentation and in frequently fermenting dextrin and starch Several biochemical reactions are of special importance for the differentiation of these two organisms (Tables 41 and 42) *A aerogenes* does not produce indol In dextrose broth it produces less acid than *E coli* as shown by the methyl red test and produces acetylmethylcarbinol which is responsible for a positive Voges Proskauer reaction *A aerogenes* is able to utilize sodium citrate as the sole source of carbon in a synthetic medium while *E coli* produces very little if any growth in such a medium These 4 differential criteria (the production of indol methyl red and Voges Proskauer tests and citrate utilization) are sometimes referred to as I M Vi C group (Table 42)

TABLE 42 DIFFERENTIAL REACTIONS FOR COLIFORM ORGANISMS

ORGANISMS	INDOL	M R	V P	CITRATE MEDIUM
E coli	+	+	—	—
A aerogenes	—	—	+	+
Kl pneumoniae	—	—	+	+

When coliform bacilli are classified on the basis of the I M Vi C tests some organisms give reactions which are intermediate between those of *E coli* and *A aerogenes* e g Indol+ MR+ VP− Citrate+ and other different combinations These organisms are known as intermediates Their occurrence suggests that in spite of the classic differences described coliform organisms are best considered as a closely related and intergrading group in which variant forms occur as a result of loss acquisition and perhaps recombination of certain characteristics *A aerogenes* may cause in man urinary tract infections which respond to the same therapy as infections due to *E coli* Differential reactions for coliform organisms are presented in Table 42

PARACOLON BACILLI

The organisms grouped together under the term paracolon bacteria ferment lactose only after prolonged incubation or not at all and thus lie outside the classic coliform group in which the immediate fermentation of lactose is a basic characteristic They differ from salmonella organisms in their slow fermentation of lactose or sucrose and the lack of the characteristic antigenic patterns and pathogenic properties of these organisms The paracolon bacteria lie between the normal coliform bacteria and the salmonella and provide an example of the intergrading relationships in the enteric group

The presence of paracolon bacilli in the intestine complicates the examination of fecal specimens for organisms of pathologic significance since they possess various combinations of the several characteristic reactions on which the differential isolation technics for pathogens and nonpathogens are based The organisms within the group are markedly heterogeneous in their biochemical and antigenic properties

Paracolon bacilli seem to occur in feces more commonly during outbreaks of gastroenteritis (Stuart et al 1943) Epidemiologic findings suggest that the Arizona the Bethesda and the Providence groups contain organisms which appear to be the cause of gastro enteritis in man These bacteria may contain one or more of the somatic antigens found in the salmonella organisms a fact which adds to the confusion in their identification and to the difficulties in the assessment of their significance as human enteric pathogens

KLEBSIELLA PNEUMONIAE AND THE FRIEDLANDER GROUP

The organisms of the Friedlander group (synonyms pneumobacillus *Bacterium friedlanderi* *Bacillus mucosus capsulatus* Friedlander s bacillus) are short nonmotile nonsporing gram negative bacilli which characteristically possess large capsules They produce a profuse mucoid growth on agar media and ferment a number of carbohydrates with production of acid and gas They are closely related to the aerobacter group They are found in the nose the mouth and the intes

tinal tract of normal persons in the lungs of patients with pneumonia and other respiratory diseases, and in suppurative infections in other parts of the body

Klebsiella pneumoniae is the most important member of the group It was discovered in 1883 by Friedlander in the lungs of patients dying with pneumonia and is now known to cause a small proportion of the bacterial pneumonias

Morphology

In infected tissues *Kl pneumoniae* usually occurs as an ovoid rod from 2 to 5 μ long and 0 5 μ thick often in pairs In cultures it shows pleomorphism with curved rods long filaments and other forms It occurs naturally in the mucoid phase with a capsule which is usually visible even in an ordinary Gram stain and is particularly striking when the organisms are grown on media rich in carbohydrate A profuse mucoid growth of a tenacious character is produced on solid media It is luxuriant and viscous in broth After repeated subculture *Kl pneumoniae* tends to lose its mucoid character and dissociates to give smooth colonies made up of organisms which do not produce the characteristic large capsules Reversion to the mucoid form may occur on further subculture

Biologic Characteristics

Kl pneumoniae is facultatively anaerobic and grows best at 37° C with a range from 15° to 40 C Growth is luxuriant on ordinary nutrient media The organisms are killed by moist heat a 55° C in 30 minutes

The biochemical reactions are variable from strain to strain These variations may occur within a single serologic type and increase the difficulty of classification *Kl pneumoniae* ferments glucose maltose lactose sucrose mannitol and salicin with the production of acid some strains fail to produce gas Indol is not produced The organism is usually MR− VP+ and does not produce H S

The morphologic and biochemical properties of *Kl pneumoniae* show that it is closely related to *E coli* from which it is chiefly differentiated by its respiratory habitat, its etiologic role in certain cases of pneumonia in man and its characteristic possession of an easily visible capsule The differentiation from *A aerogenes* is much more difficult It becomes indistinguishable from other coliform organisms when it undergoes dissociation from the mucoid to the smooth form and thereby loses its large capsule (Ostermann and Rettger 1941)

The antigenic structure of *Kl pneumoniae* has been studied by Julianelle (1926) who found that the capsule contains a type specific polysaccharide He described 3 serologic types A B C and a fourth heterogeneous group X The specific polysaccharide of the type B organism shows an immunologic relationship to that of the type 2 pneumococcus A nucleoprotein which is common to all types is found in the body of the bacterial cell

In more recent studies Kauffmann (1949) has identified somatic (O) antigens in *Kl pneumoniae* which permit the classification of this organism into 3 principal antigenic O groups These O antigens also occur in *A aerogenes* indicating again their close relationship In addition he has extended the studies of the capsular K antigens to describe a total of 14 types The same capsular antigen may be found in organisms in different O groups

Pathogenicity

Kl pneumoniae is found in the respiratory tract of about 5 per cent of normal individuals (Bloomfield 1921) and frequently occurs as a secondary invader in the lungs of patients with bronchiectasis influenza and tuberculosis It is the primary cause of pneumonia in less than 3 per cent of all bacterial pneumonias (Hyde and Hyde 1943) It has been isolated from patients with pleurisy appendicitis cystitis and pyelonephritis and from the feces of about 5 per cent of normal individuals (Baehr et al 1937) It occurs frequently in abdominal infections though its presence is often overlooked in the bacteriologic studies

In animals *Kl pneumoniae* has been isolated from spontaneous respiratory diseases of mice and in a metritis occurring in mares Types A and B are highly pathogenic for mice by intraperitoneal injection while guinea pigs and rabbits show a higher degree of resistance Type C appears to be relatively avirulent for animals

In pneumonic infections in man *Kl pneumoniae* is notable for its destructive action on the tissues, producing abscesses and cavi

ties Julianelle (1941) found Type A in 64 per cent Type B in 14 per cent Type C in 7 per cent and Group X types in 15 per cent of a series of cases of pneumonia The mortality of untreated cases is high and may reach 90 per cent in those with bacteremia Chronic Friedlander bacillus infections of the lung may follow the acute pneumonia with production of cavities and thus require surgical intervention

Sulfadiazine and streptomycin have proved to be of value in therapy as well as chloramphenicol chlortetracycline and oxytetracycline Two or more drugs are usually employed for maximal therapeutic effectiveness

Two other encapsulated mucoid gram negative bacilli resembling *Kl pneumoniae* and occurring characteristically in the mucoid phase have been isolated from disease conditions of the upper respiratory tract of man Frisch in 1882 isolated an encapsulated organism from the granulomatous nasal lesions of patients with rhinoscleroma and a similar organism was isolated by Loewenberg in 1894 from the nasal secretions of individuals with ozena a fetid catarrhal condition of the nose The capsular antigen of the rhinoscleroma organism is similar to that of Type C Friedlander s bacillus but *Kl ozenae* may be easily differentiated from *Kl pneumoniae* and *Kl rhinoscleromatis* by serologic tests

Logically the salmonella and shigella groups should be discussed at this time as they are closely related to the coliform bacilli However they will be considered in Chapters 16 and 17 respectively

THE PROTEUS GROUP

Introduction

The proteus group consists of pleomorphic gram negative bacilli which do not ferment lactose and are characterized by their active motility and spreading growth of solid media They are commonly found in soil water sewage and manure and occur in normal human stools They are not usually pathogenic for man though they cause infections of the genito urinary and the gastro-intestinal tracts The two commonest pathogenic species are *Proteus vulgaris* and *Proteus morgani* other strains are of medical importance because of their antigenic relationships to certain rickettsiae

Proteus Vulgaris

Proteus vulgaris is a motile gram negative rod shaped organism which is subject to great variation in size and shape The more typical forms in agar cultures average from 1 to 3 μ long and from 0 4 to 0 6 μ wide but short coccobacillary forms are also seen The rod occur singly in pairs and frequently in long chains Young cultures which show swarming are particularly pleomorphic and may include long filamentous forms

The organism is a facultative anaerobe with an optimum growth range from 34 to 37 C but able to grow well at 20 C on solid moist media It spreads rapidly from the initial colonies over the entire surface by a process called swarming which is due to the very active motility of the bacilli Swarming may be prevented by increasing the agar content of the media to 6 per cent In broth the organism gives a moderate uniform turbidity with some deposit

Proteus vulgaris produces acid and gas in glucose sucrose and galactose Some strains ferment maltose The maltose fermenting strains which form indol are VP negative and usually fail to grow on citrate agar while those which do not ferment maltose are indol negative usually VP positive and usually grow on citrate agar *P vulgaris* exhibits active proteolytic action and liquefies gelatin digests casein and decomposes urea It produces H S NH_3 and reduces nitrates

The antigenic structure of *Proteus vulgaris* has received considerable attention because of the use of certain strains designated by the letter X in the Weil Felix reaction for the diagnosis of typhus fever and of other rickettsial diseases The group is antigenically heterogeneous with differences in both H and O antigens On the basis of their serologic relationships to certain rickettsia the O antigens of the X strains have been divided into 3 types OX2 OX19 and OXK Castaneda (1934) reported the isolation from Proteus OX19 of a soluble specific polysaccharide which seems to be present also in *Rickettsia prowazeki* and to be responsible for the serologic reaction utilized in the Weil Felix test in epidemic typhus fever

Organisms of the Proteus group are widely distributed in nature They are found in water and soil and form an important part of the flora of decomposing animal and vegetable matter in manure and sewage The organisms occur in the feces of man and animals but in large numbers only when some abnormal condition exists They become more prominent when stool specimens are incubated in the enrichment media tetrathionate broth or selenite F used for suppressing *E coli* in the isolation of enteric pathogens In addition to its saprophytic existence *P vulgaris* may be isolated in pure or mixed cultures in urinary tract infections from abscesses or wounds and in peritonitis It causes up to 13 per cent of human urinary tract infections (Pierson and Honke 1941) and often appears in patients who have been treated successfully with various antibacterial agents for infections caused by other genito urinary pathogens It appears in large numbers on infected wounds after antibiotic therapy by reason of its ability to develop drug resistance and thus to overgrow the drug sensitive species *P vulgaris* has been isolated from cases of gastro enteritis where it appeared to play an etiologic role (Cooper et al 1941) Isolation of the X strains of *Proteus* from the urine and the feces of patients with typhus fever led to the study of the possible (now discounted) etiologic relationship to typhus and to the discovery and the use of the Weil Felix reaction

The intraperitoneal inoculation of *Proteus vulgaris* into mice rats guinea pigs or rabbits often causes death of these animals but strains vary greatly in pathogenicity

The sulfonamides are of limited value in the treatment of infections caused by *Proteus vulgaris* Streptomycin has proved to be useful in some cases but these organisms readily become resistant to it Chloramphenicol is effective in certain cases

Proteus Morgani

Proteus morgani (Morgan s bacillus) was isolated by Morgan in 1906 from the stools of patients with diarrhea Most strains show the swarming characteristics of the Proteus group usually at lower incubation temperature e g 20 C but tend to lose this property on cultivation Likewise motility is pronounced at room temperature but it decreases or it is lost at 37° C Although Morgan s bacillus is related closely to *P vulgaris* as shown by its ability to swarm and to decompose urea, it does not liquefy gelatin or produce H S It produces indol and ferments for the most part only monosaccharides

Proteus morgani has been isolated on a number of occasions from outbreaks of infantile diarrhea (Neter and Farrar, 1943) where it seemed to play an etiologic role but it is difficult to assess the significance of these findings, since it has also been isolated from the stools of normal persons This organism may cause genito urinary tract infections in man and, rarely purulent lesions of other areas Its role as a potential cause of enteric fever in man has been suspected but not clearly proved Spontaneous epidemics of enteritis due to it have been observed in mice

MISCELLANEOUS GRAM NEGATIVE BACILLI

Pseudomonas Aeruginosa

The pseudomonas group is composed of gram negative, rod shaped, motile organisms which characteristically produce a water soluble pigment which diffuses through the medium They occur widely in water and soil Some species are pathogenic, and the type species *Pseudomonas aeruginosa* (*Pseudomonas pyocyanea Bacillus pyocyaneus*) occurs in human feces and in wound and urinary tract infections in man

Gessard in 1882 isolated *Ps aeruginosa* from the blue pus found in some wound infections This organism is closely related to about 30 other species of *Pseudomonas* which occur principally in soil water and sewage although some produce disease in animals and plants *Ps fluorescens* is one of the most common of these other species

Ps aeruginosa is a gram negative, motile rod measuring from 1 5 to 3 0 μ by about 0 5 μ It is not encapsulated and forms no spores It grows readily on all ordinary culture media and has a sweetish odor On agar it forms round smooth moist glistening colonies which have a fluorescent yellowish green color although most of the pigment diffuses into the medium coloring it bluish green The organism is aerobic and grows best at

30 to 37° C It is killed at 55 C for 1 hour

It is not an active fermenter of carbohydrates and produces acid but no gas in glucose It actively liquefies gelatin produces ammonia and grows on citrate medium *Ps aeruginosa* does not produce indol is methyl red and Voges-Proskauer negative it fails to produce hydrogen sulfide and to reduce nitrates

The bluish green pigment produced by *Ps aeruginosa* consists of two substances pyocyanin a bluish green pigment soluble in chloroform and water and fluorescin which is greenish yellow fluorescent and soluble in water but not in chloroform The closely related organism *Ps fluorescens* forms only fluorescin These pigments are antibacterial for certain other organisms

Ps aeruginosa is the only member of this group pathogenic for man It is found occasionally in the human intestine and on the skin as well as in water and sewage It may produce local suppurative lesions especially skin and wound infections and otitis media It may occur in infections of the genito-urinary tract the respiratory tract the joints and the eye Meningitis due to it has been observed to follow a lumbar puncture or operative exposure of the meninges In some outbreaks of a dysenterylike enteric infection *Ps aeruginosa* has been isolated under circumstances which suggest an etiologic role

With the more frequent use of antibiotics that eliminate many of the pathogens in respiratory and urinary tract infections *Ps aeruginosa* has assumed more importance etiologically since it emerges as the dominant organism when the more susceptible species disappear *Ps aeruginosa* may cause pneumonia in debilitated individuals receiving prophylactic penicillin or may appear as the causative agent in chronic pyelonephritis when other organisms have been eliminated by proper therapy It may also become the predominant organism on the injured skin of a burned patient receiving antibiotic therapy and give a blue green color to the surface exudate

It produces fatal infections when injected subcutaneously or intravenously into guinea pigs or rabbits

Streptomycin chlortetracycline and chloramphenicol have proved to be of some value in treating infections by *Ps aeruginosa* but polymyxin appears to possess greater therapeutic potency for this organism

Alcaligenes Faecalis

Alcaligenes faecalis is a gram negative rod which is found in human feces and can be confused with *Salmonella typhosa* since it does not ferment lactose and therefore produces similar colonies on the usual differential media used for the isolation of enteric pathogens It is readily distinguished from other gram negative organisms by its failure to ferment glucose as well as most other carbohydrates In rare instances it may cause enteric infections in man and is not uncommon as a cause of inflammation in the urinary tract

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16

The Salmonella

INTRODUCTION

The salmonella are gram negative nonspore forming motile bacilli easily cultivated on ordinary media and they characteristically fail to ferment lactose and sucrose The different species are closely related antigenically and these relationships are used as the main criterion in classification All are pathogenic for man or animals and usually for both *Salmonella typhosa* the cause of typhoid fever is pathogenic only for man while the other salmonella produce disease in man and animals

HISTORY

Before the development of modern bacteriology and the isolation of the typhoid bacillus William Budd in 1856 made a strikingly accurate study of typhoid fever outbreaks which led him to believe that the disease was contagious and that the infectious agent was excreted in the feces of patients He believed that contaminated milk and water played a role in the spread of the disease In 1880 Eberth described the typhoid bacillus in tissues of patients and the organism was isolated by Gaffky in 1884 Following this other Salmonella were isolated from patients with typhoidlike fevers and it became obvious that a clinical syndrome similar to typhoid fever might be caused by a variety of closely related organisms

Gaertner in 1888 isolated *Salmonella enteritidis* from a patient who died following the consumption of meat contaminated with this organism and shortly afterward Durham and de Noeble described another organism *S typhimurium* isolated from patients suffering with gastro-enteritis following ingestion of infected meat Further studies rapidly increased the numb rs of organisms identified as having the typical properties of members of the Salmonella group

MORPHOLOGY

Salmonella average from about 2 to 3 μ in length and about 0 6 μ in width but may show variation in size under different environmental conditions Young cultures on agar may present a predominance of coccobacillary organisms while filamentous forms are occasionally seen especially in cultures in liquid media With the exception of *S gallinarum pullorum* all strains are motile with peritrichal flagella Salmonella do not ordinarily form capsules when grown at 37 C but most species may give rise to mucoid colonies composed of encapsulated bacilli e pecially when incubated at temperatures of 20° C or lower Encapsulated strains of *S typhosa* have been described

CULTIVATION AND BIOCHEMICAL REACTIONS

Salmonella grow readily on ordinary culture media producing in 24 to 48 hours colonies which average 2 to 3 mm in diameter and are indistinguishable from those of the coliform bacteria The colonies may be circular with a smooth surface and an even edge or

flat with an uneven surface and serrated edge On plates incubated at 37° C and then allowed to stand at room temperature, they may show a secondary growth of mucoid character around the original colony margin Salmonella in the smooth phase produce a uniform turbidity in broth with a deposit which readily resuspends on shaking They may produce heavy growth under anaerobic conditions The temperature range of growth is from about 10 to 42° C with an optimum at 37° C

The biochemical reactions serve to define the salmonella as a group and also provide an aid in the differentiation of certain species (Table 41) By definition these bacteria do not ferment lactose sucrose or salicin while glucose mannitol maltose and dextrin are fermented with the production of acid and gas except in the case of *S typhosa* and *S gallinarum pullorum* which do not form gas Arabinose xylose trehalose and inositol are useful in the differentiation of certain species and varieties e g *S paratyphi* does not ferment xylose while *S schottmuelleri* does The fermentation of tartrate varies with different species while almost all attack citrate Indol is not produced and gelatin is not liquefied since there are very few exceptions to these two metabolic activities they serve along with the failure to ferment lactose as the most reliable biochemical differential characters

EFFECTS OF PHYSICAL AND CHEMICAL AGENTS

Most of the salmonella are killed at a temperature of 60° C in from 15 to 20 minutes They may persist under natural conditions for long periods of time as demonstrated in soil and water pollution studies in which typhoid bacilli have been found to survive through an entire winter in frozen soil and for as long as 7 days in well water The resistance of salmonella to certain dyes and chemicals is important since some of these compounds selectively inhibit the coliform organisms while allowing growth of the salmonella Brilliant green in particular inhibits coliform and dysentery bacilli while the typhoid bacillus and other salmonella are resistant to its action Sodium desoxycholate and selenium compounds also inhibit the growth of colon bacilli but not the salmonella under certain conditions Sodium tetrathionate and sodium citrate favor the growth of salmonella over colon bacilli The selective action of these compounds is used to advantage in the preparation of media for the isolation of salmonella from feces

ANTIGENIC STRUCTURE

The antigenic structure of the salmonella has been studied in great detail by Kauffmann (1937) and White (1926) As a result of their work, the various known antigenic components in the cell body and the flagella now constitute the basis of classification The H or flagellar antigens and the O, or somatic, antigens were described originally as the heat labile and the heat stable antigenic components respectively In general the species of *Salmonella* are divided into groups on the basis of likeness with respect to O or somatic antigens and the species within a group are often differentiated on the basis of differences between their H or flagellar antigens (Kauffmann 1950) Some of these species also possess an envelope antigen called Vi (for virulence) which inhibits agglutination by O antibody

H Antigens and Phase Variation

The H antigens are found only in the flagella They are inactivated by temperatures over 60° C and also by alcohol and acids They are probably of a protein nature For serologic testing H antigens are best prepared by adding formalin to young motile broth cultures This procedure probably fixes the flagella over the surface of the cell in such a way that the somatic antigens are no longer exposed As a result agglutination is dependent on the anti H antibodies and does not occur or only to a slight degree in anti O sera In sera containing the appropriate anti H antibodies H antigens characteristically flocculate in about 2 hours at 55° C in the form of large fluffy clumps which are easily dispersed

A single species may contain two types of H antigens either of which may predominate in a given instance One of these types is referred to as the specific phase or phase 1 flagellar antigen and the other as the group phase or phase 2 flagellar antigen The former is shared with only a few other species or

varieties of *Salmonella* In contrast the latter may be more widely distributed among several species Either phase 1 or phase 2 may contain one or more flagellar antigenic components Any one culture may consist of organisms *entirely* of one phase or of organisms in both flagellar phases Any monophasic culture usually tends to maintain this characteristic for a number of transfers but is always capable of giving rise to organisms of the other phase especially if the culture is allowed to grow longer than 24 hours This antigen alteration is spoken of as phase variation The transformation from one phase to another in a culture may be induced by growth of the culture in a serum containing antibodies against the homologous phase Since the specific phase antigens are not entirely limited to one *Salmonella* species but may occur in several the terms phase 1 for the so-called specific phase and phase 2 for the group phase have now been adopted Phase variation can be detected only by serologic tests using sera prepared against organisms in phase 1 or phase 2

O Antigens

The somatic (O) antigens do not exhibit *phase variation and therefore constitute a* more dependable basis for classification than the flagellar components (Kauffmann White schema) Somatic antigens occur at the surface of the cell body (soma) in both motile and nonmotile organisms They are resistant to prolonged heating at 100 C and are not destroyed by alcohol or dilute acid When mixed with sera containing appropriate anti O agglutinins O (somatic) antigens (prepared from nonmotile bacilli or from bacilli treated with heat or alcohol) are clumped only after long periods of incubation e g 6 to 12 hours at 55 C The bacterial aggregates so formed appear as granular masses which cannot be *dispersed by shaking*

In an antiserum prepared with a motile organism as immunizing agent the anti H and the anti O agglutinins behave independently and the H antibody titer is usually much higher than the O

Vi Antigens

Recently isolated strains of typhoid bacilli often fail to agglutinate in antiserum Felix and Pitt (1934) showed that this inagglutinability is due to a special somatic component called the Vi or virulence antigen since cultures possessing it were more virulent for mice than ordinary O organisms Vi antigen is thought to be present at the extreme periphery of the cell as an envelope antigen and thus prevents access of anti O agglutinins to their homologous somatic antigens It differs from the ordinary O antigens in being destroyed by heating for one hour at 60 C and by dilute acids and phenol Following repeated subculture on ordinary media bacilli lose their Vi antigen and become agglutinable in anti O serum A Vi antigen apparently identical with that found in *S typhosa* has also been recognized in other Salmonella e g *S hirschfeldii* and *S ballerup* Other Salmonella such a *S schottmuelleri* possess other Vi antigen specific for their species

In 1938 Craigie and Yen discovered bacteriophages active against cultures of the typhoid bacillus containing Vi antigen These agents exhibit an unusual adaptability to the strains of bacteria on which they are propagated resulting in the development of an extraordinary specificity for the particular strain of *S typhosa* Specific Vi phages have *thus provided a valuable means of classifying* typhoid bacilli and have aided etiologic studies of typhoid fever Later Anderson and Felix (1953) presented evidence that the phage type is an expression of resistance to infection with the typing phages caused by latent phages in the bacteria being tested Strain specific bacteriophages have also been adapted for use in identifying strains of *S schottmuelleri*

DISSOCIATION

In addition to the flagellar phase variations salmonella can undergo a number of alterations related to various antigenic components Motile strains may become nonmotile by losing their flagellar (H) antigens The typical smooth to rough variation occurs frequently and results in the loss of the somatic O antigen and the appearance at the surface of the cell of another antigen which is much less specific and causes the organism to agglutinate spontaneously in saline The smooth to rough *change may occur without loss of the Vi or* flagellar antigens

The Vi antigen content of any strain of *S typhosa* may vary without affecting its O

or *H* antigens Kauffmann (1935) has described 3 forms in which these organisms may be found (1) the V form, which possesses a full quota of Vi antigen and is inagglutinable in O antiserum (2) the VW form, in which some Vi antigen may be detected, but which will agglutinate in O antiserum and (3) the W form which contains no Vi antigen Since the *H* the *O* and the Vi antigens vary independently many dissociant forms can occur

As previously mentioned some salmonella also show a tendency to produce mucoid colonies especially when incubated at temperatures of from 10° to 20° C

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The studies of the salmonella antigens by White and Kauffmann have made it possible to devise a system of classification based on antigenic patterns The species and the varieties have been arranged in groups designated A B, C etc according to similarities in content of O antigens and one or more antigenic components are selected as essential for inclusion in each group Each component of the O antigen is designated by the use of a Roman numeral Specific sera to identify O antigens are prepared by absorption technics Table 43 shows how the more important representative

TABLE 43 ANTIGENIC STRUCTURE OF SOME OF THE MORE COMMON ENTERIC ORGANISMS

			H ANTIGENS	
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A	*S paratyphosa*	(I) II XII	a	
B	*S schottmuelleri*	(I) IV (V) XII	b	1 2
	S typhimurium	(I) IV (V) XII	i	1 2
C_1	*S hirschfeldii*	VI VII (Vi)	c	1 5
	S choleraesuis	VI VII	c	1 5
	S oranienburg	VI VII	m t	
	S montevideo	VI VII	g m s	
C_2	*S newport*	VI VIII	e h	1 2
D	*S typhosa*	IX XII (Vi)	d	
	S enteritidis	(I) IX XII	g m	
	S gallinarum pullorum	I IX XII		
E	*S anatum*	III X	e h	1 6

( ) indicate that antigen may be absent

Salmonella are classified according to the Kauffmann White scheme The members of the various groups based on the O antigen content are further differentiated into species and varieties on the basis of the components of their flagellar antigens The flagellar antigens of phase 1 are noted by small letters and the antigens of phase 2 by arabic numerals

The Kauffmann White system now includes over 150 types, but it is questionable whether many warrant recognition as distinct species since the antigenic relationships among them are so very close Probably many of the closely related organisms might best be considered as varieties or serologic types of a single species as has been the practice with the serotypes of *Diplococcus pneumoniae* In this connection it should be pointed out that differences in the ability to ferment one or more carbohydrates may exist between strains which apparently are antigenically identical These are designated as fermentative varieties

The scheme of classification based on antigenic analysis is complex, but it permits the accurate etiologic diagnosis of enteric infections and the study of their epidemiology

DISTRIBUTION AND RANGE OF PATHOGENICITY

Salmonella fall into 3 groups with respect to their distribution and relationship to human disease

The first group contains those which are primarily human pathogens and includes *S typhosa S paratyphi S schottmuelleri* and and *S hirschfeldii* Of these *S typhosa* is the most important In the United States *S schottmuelleri* is the most common (Selig mann et al, 1946) occasionally *S paratyphi* is isolated and *S hirschfeldii* is very rare *S schottmuelleri* is also found rarely in animals

The second group is made up of organisms which are primarily pathogenic for animals including birds but occasionally may cause disease in man It contains the majority of the salmonella The relative incidence of these species in human infections varies in different geographic areas Frequently, they are named to designate the area or the city where they were first isolated or to indicate their principal animal host In the United States the following organisms have been most commonly isolated from patients *S typhimurium S*

choleraesuis S oranienburg S montevideo S newport S enteritidis S panama and *S anatum* (Bornstein 1943) The relative incidence of infections due to any one species among the reported cases depends in large part on the number of persons involved in the outbreaks studied

In the third group are found those which are known to be pathogenic only for animals or birds This group has rapidly become smaller as more of these species have been found to cause disease in man *S gallinarum pullorum* is one of the most important organ isms in this group

Salmonella typhosa (*S typhi Eberthella typhosa Bacillus typhosa, Bact typhosum* typhoid bacillus) The typhoid bacillus is found only in man and is the cause of classic typhoid fever In animals by the oral route it will infect only chimpanzees By the intra venous or the intraperitoneal route *S typhosa* in large doses will kill mice but the size of the dose indicates that its invasive powers are not marked and that death in this host is mainly a result of the toxic action of the organisms injected The addition of mucin to the suspen sion of bacteria enhances the ability of the bacillus to multiply in the mouse and makes it possible to produce death with much smaller numbers of organisms

Salmonella paratyphi (*Salmonella para typhi 1 Bacillus paratyphosus A Bacterium paratyphosum A* paratyphoid A bacillus) is found only in man and is one cause of para typhoid fever

Salmonella schottmuelleri (*Salmonella para typhi B Bacillus paratyphosus B Bacterium paratyphosum B* paratyphoid B bacillus) is also a cause of paratyphoid fever in man and in addition produces gastro enteritis Although occasionally it has been isolated from animals it does not cause disease in them under natural conditions

Salmonella hirschfeldii (*Salmonella para typhi C*) is a common cause of enteric fevers or gastro enteritis in man in Eastern Europe and Asia but is rarely found in the United States It is not known to be a natural patho- gen of animals

Salmonella typhimurium (*Salmonella aert rycke Bacterium aertrycke*) is a natural pathogen of rodents causing in mice a ty phoidlike disease which has a high mortality It usually produces an acute gastro enteritis in man

Salmonella choleraesuis (*Bacterium suipes tifer*) though a natural pathogen for animals may cause an enteric fever or gastro-enteritis in man but usually produces localizing proc esses with or without a septicemia *S chol eraesuis* also occurs in the intestine of normal hogs and occasionally in cattle and sheep It is a prominent secondary invader in hog cholera and was once considered to be the etiologic agent until the real cause a filterable virus was isolated Contaminated meat may be a source of infection

Salmonella oranienburg occurs naturally as a cause of epizootics in quail and chickens and frequently is isolated from dried egg products In man it may produce gastro-enteritis enteric fever or septicemia

Salmonella montevideo is found in various animals and fowls (monkeys pigs turkeys chickens etc) and also is isolated from dried egg products In man it may cause gastro- enteritis enteric fever or a septicemia

Salmonella newport is isolated from rats pigs chickens and turkeys as well as from meat and dried eggs It causes gastro-enteritis enteric fever or septicemia in man

Salmonella enteritidis (*Bacterium enteri tidis*) which resembles *S typhimurium* in many properties was one of the first salmo nella identified as the cause of disease in man by Gaertner in 1888 It includes several fer mentative varieties which sometimes are given separate designations such as *S enteritidis* var *gartner danyz* or *essen* Some relation ship between these varieties and a specific natural host seems to exist Strains have been isolated from horses hogs mice rats ducks and duck eggs In man it causes gastro- enteritis more often than other types of clini cal manifestations

Salmonella anatum isolated on several occasions from ducks chickens turkeys and dried eggs has also been found in normal pigs and silver foxes In man it u ually pro- duces a gastro-enteritis

Salmonella gallinarum is the cause of fowl typhoid and differs from other salmonella in being nonmotile An important fermentative variant *S pullorum* is the cause of bacillary white diarrhea in chickens and is found in dried egg products These organisms are com monly regarded as nonpathogenic for man though recent evidence has shown that some times *S pullorum* may cause gastro-enteritis when ingested in large numbers

From this abridged list of representative salmonella it is apparent that most of the strains that are natural pathogens of animals can also produce disease in man Animal products such as meat milk or eggs are often

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number of polymorphonuclear leukocytes and an absence of eosinophils

Blood cultures are often positive in the first and the second weeks and less frequently during the third week stool cultures may be positive from the beginning and often remain positive until convalescence has been completed Organisms are often found in the urine during the second and the third week and may be excreted for a considerable period after convalescence (Fig 36) Cultures of the bone marrow aspirations may show typhoid bacilli when blood cultures are negative Organisms may also be found in the rose spots

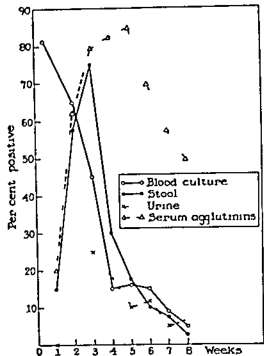


FIG 36 Results of serum agglutination tests and of blood stool and urine cultures on patients during the course of typhoid fever

The typhoid bacillus gains access to the body through the alimentary tract where it probably multiplies during the incubation period in the lymphoid tissue of the wall of the small intestine and the regional lymph nodes Later organisms are often found here in the plasma cells The intracellular position of the organism may account for the persistence of the infection in the presence of bactericidal antibodies in the blood serum and for the failure of therapeutic immune serum to alter appreciably the clinical course of the disease It is probable that the bacilli enter the blood stream from these cells at the end of the incubation period via the lymphatics and the thoracic duct Then the endotoxins are released in the blood stream as the bacterial cells are lysed producing some of the symptoms of typhoid fever There is some evidence that the somatic antigens are present in the blood of patients during the acute phase of the disease (Dennis and Saigh 1946)

Typhoid bacilli are found frequently in the spleen and bone marrow and the gallbladder is invariably infected The organisms appear to multiply in the biliary system and the majority of those which are found in the stool at certain stages of the disease particularly in convalescence probably are carried there with the biliary secretions The periodicity of the discharge of the biliary contents into the intestine may account in part for the irregular results in the isolation of *S typhosa* from the feces of any given patient over a period of time

Relapses occur in about 10 per cent of the cases and probably represent a reinvasion of the blood stream from the local areas of multiplication of the organisms in the lymphoid tissue bone marrow the spleen and the biliary system The mortality rate in typhoid fever is about 10 per cent and death is due to the complications of intestinal hemorrhage or perforation in about 75 per cent of the fatal cases

On postmortem examination the small intestine usually shows extensive areas of ulceration particularly in the area of Peyer s patches and hyperplasia of lymphoid tissue in the intestinal wall Many organisms are present in the lymphoid tissue some within plasma cells The spleen is enlarged The liver shows areas of focal necrosis and organisms are almost always present in the gallbladder Other organs may be involved such as the periosteum bone marrow joints and lungs In patients with signs of meningitis occasionally *S typhosa* has been isolated from the spinal fluid

OTHER ENTERIC FEVERS

Enteric fevers caused by salmonella other than *S typhosa* have a shorter incubation

the vehicles involved in transmission to man These foods may come from infected animal sources or be contaminated by infected animals or man before ingestion

TOXINS

The *endotoxins* of the salmonella are materials closely associated with the bacterial body which are released in solution only by autolysis and appear to be identical with the cell components mentioned earlier under the name of somatic antigens They are heat stable and neutralized only to a slight degree by immune sera The studies of Boivin et al (1933 a b) and Henderson and Morgan (1938) have revealed the presence in Salmonella of a polysaccharide protein lipid complex which seems to be identical with the somatic antigen of the cell and possesses all of the properties ascribed to the endotoxins Somatic antigens isolated from the various species of *Salmonella* are similar in chemical nature The polysaccharide is responsible for the serologic type specificity of the antigen whereas the protein seems to be antigenically identical to the various salmonella and shigella The purified somatic antigen complexes elicit the production of O agglutinins bactericidal antibodies and mouse protective antibodies when injected into rabbits or man (Morgan 1941 Favorite and Morgan 1942)

Somatic antigens are highly toxic Given intravenously in man they produce a marked febrile response This toxic effect is not completely neutralized by specific immune serum (Morgan 1941) Intradermal inoculation into rabbits and man produces local edema and erythema followed by necrosis Intravenous injection into rabbits produces congestion hemorrhagic extravasation and necrosis in various organs particularly the liver and bone marrow The latter shows very few polymorphonuclear leukocytes The vascular endothelium is damaged and widespread thrombosis similar to the type characteristic of severe typhoid infections in human beings is produced (Morgan 1943) The changes in the bone marrow and the focal necrosis in the liver are also similar to those observed in fatal human cases of typhoid fever Following the intravenous injection in man of minute amounts of the purified somatic antigens the individual develops a chill fever headache malaise and a polymorphonuclear leukopenia These symptoms and the *leukopenia are common* manifestations of typhoid fever in man

A local intradermal injection of the somatic antigens followed after an interval by an intravenous injection results in the local hemorrhagic changes in the skin characteristic of the Shwartzman reaction

PATHOGENESIS

Salmonella infection is almost always due to the ingestion of contaminated materials the organisms enter the tissues from the intestine via the lymphatics Since the number of organisms ingested may affect the length of the incubation period the latter may vary considerably in different individuals

There are 3 main types of clinical manifestations of Salmonella infection, namely enteric fever gastro enteritis and a localizing type with foci in one or more organs accompanied by septicemia

TYPHOID FEVER

Among the enteric fevers the classic example is typhoid fever The typhoid organism unlike other salmonella produces only this clinical manifestation in the human host The incubation period extends from about 7 to 14 days The onset is insidious often beginning gradually with malaise anorexia and a headache This usually is followed by the appearance of a fever which rises in a steplike manner to an average of 104° F with a pulse rate that tends to be slow in comparison with the height of the fever Nosebleeds may occur at this stage of the disease During the first week the patient usually is prostrate and may have diarrhea though constipation is even more common and either is usually accompanied by abdominal tenderness and distension At this time the patient may also have a cough and bronchitis In the first or the second week rose spots frequently appear splenomegaly is common and the temperature remains elevated In the more severe cases as time passes the patient may become delirious and show the so called typhoid state for which the disease was named After the third week the temperature curve shows morning remission and returns to a normal level by a gradual lysis A leukopenia is present in most cases characterized by a relative decrease in the

et al 1946) 28 different Salmonella types were isolated with *S schottmuelleri S typhimurium S oranienburg S montevideo S newport S panama* and *S anatum* being the most common Many of these carriers were contacts of cases and had exhibited no clinical symptoms of infection

IMMUNITY

An attack of typhoid fever usually confers immunity though second attacks have been reported Recovery from the disease is associated with the appearance in the blood of agglutinins and bactericidal antibodies for *S typhosa* These antibodies reach appreciable levels during the second and the third weeks of the disease at a time when the typhoid bacilli are known to disappear from the blood stream However antibodies may be present during the acute phase of the disease in relapses and at the time of progression to fatal termination (Gay 1918) These facts strongly suggest that the presence of known antibodies is not the sole factor in recovery

It seems probable that the circulating antibodies clear the extracellular organisms from the blood but that the bacilli which are known to occur intracellularly in the spleen the gall bladder bone marrow and the lymphoid tissue of the intestine are protected from their action The e bacilli multiply within the cells and release the somatic endotoxins which seem to produce tissue damage and the symptoms of general toxemia Since the toxicity of these substances is only reduced and not completely neutralized by antibodies they may produce their injurious effects in the presence of circulating antibody (Morgan 1941) Therefore humoral antibodies appear to have a limited role in immunity to typhoid fever and the decisive factor in recovery may be the development of an increased capacity of the fixed phagocytic cells to destroy the bacteria The persistence of resistance after antibodies have disappeared from the blood tends to support this hypothesis

Another factor of possible importance in recovery from the disease may be the development of an increased tolerance to the toxic effects of the somatic antigens not dependent on the presence of antibodies (Neva and Morgan 1950) In man increasing doses of typhoid somatic antigen may be given intravenously without corresponding increases in the severity of successive reactions (Favorite and Morgan 1942) This tolerance does not seem to be correlated with antibody titer since relatively small amounts of toxin may produce toxic effects in the presence of high levels of circulating antibody Furthermore tolerance disappears fairly rapidly after the injections of toxin are discontinued while the antibody levels remain elevated for much longer periods This type of tolerance may involve a change in the functional activity of the reticuloendothelial system providing for a more rapid disposal of foreign material (Beeson 1946) It is not specific for a particular somatic antigen but appears to extend to the chemically and toxicologically related but immunologically distinct endotoxins of other gram negative organisms (Morgan 1948 a b)

IMMUNIZATION

Within a few years after the isolation of *S typhosa* suspensions of killed bacilli were injected into human beings for the purpose of immunization Adoption of this procedure in military personnel was accompanied by a marked decrease in mortality due to typhoid fever (Table 44) Although available data tend to support the view that vaccination significantly reduces the incidence of this infec

TABLE 44 MORTALITY FROM TYPHOID FEVER

PERIOD	UNITED STATES (Rates per 100 000)	UNITED STATES ARMY (Rates per 100 000)
1906 1910	25 6	26 3[illegible]
1911 1915	16 6	3 24*
1916 1920	11 1	5 08†
1921 1925	7 6	0 4
1926 1930	5 1	1 19
1931 1935	3 5	0 59
	93 CITIES‡ IN THE UNITED STATES	UNITED STATES ARMY IN THE EUROPEAN THEATER§
1942 1945	0 25	0 06

* 1911—Compulsory immunization introduced into U S Army

† 1916—Mexican Border Service 1917 1919 World War I

‡ Total population of 38 060 662

§ Army had maximum strength of 3 064 567 in this theater

period from 1 to 10 days, and, with the exception of some of the cases due to *S paratyphi S schottmuelleri* and *S hirschfeldii* are milder and less typical than typhoid fever Fever and malaise are the dominating symptoms and last from 1 to 3 weeks Blood cultures are often positive early in the disease, while stool cultures may be negative for 1 or 2 weeks Rose spots are rare The postmortem findings may or may not be similar to those of mild typhoid fever *S schottmuelleri* is a most common cause of enteric fever in the United States

GASTRO ENTERITIS

Following the consumption of contaminated food gastro enteritis caused by salmonella occurs after an incubation period of from 8 to 48 hours This short interval suggests that large numbers of the organisms are usually ingested The onset is nearly always sudden and may be characterized by headache, chills and often abdominal pain Nausea, vomiting and diarrhea follow with a rise in temperature prostration develops which lasts from 1 to 4 days The disease is more severe in infants and young children Blood cultures are usually negative but frequently organisms can be isolated from the feces and occasionally from the vomitus *S typhimurium* is the organism most commonly isolated from cases with salmonella gastro enteritis in the United States

SEPTICEMIAS

In the septicemias caused by salmonella the invasion of the blood stream is evidenced by the high remittent fever and positive blood cultures Intestinal involvement is usually absent in adults although in children the septicemia may occur as a complication of gastro enteritis Organisms may localize in any tissue of the body and may produce local abscesses in the perineal and the pelvic regions, cholecystitis pyelonephritis endocarditis pericarditis, meningitis arthritis or pneumonia *S choleraesuis* is one of the most common organisms found in this particular type of infection The mortality in salmonella septicemia averages about 5 per cent but may reach 20 per cent in infections with *S choleraesuis* (Seligman et al 1946)

Every *Salmonella* strain is potentially capable of producing any of these 3 clinical types of infection, though *S paratyphi* and *S schottmuelleri* tend more frequently to cause the enteric fever picture This potentiality may be manifested in the clinical course of disease in a single patient such as onset of illness as acute gastro-enteritis and, after temporary improvement, the development of an enteric fever due to the same organism (e g *S schottmuelleri*) *S typhimurium* and *S enteritidis* are found predominantly in acute gastro enteritis, while *S choleraesuis* is found mostly in the septicemic type of infection In all instances, infections produced by salmonella are more severe in infants and children than in adults Subclinical infections in which symptoms are absent or so mild as to cause no disability often occur with salmonella other than *S typhosa* and organisms are found in the feces In some instances, especially in gastro enteritis, more than one species of salmonella may be isolated from the stool of a single patient (Saphra and Winter 1957)

The organisms are frequently excreted in the feces during convalescence from salmonella infections or after subclinical infections and occasionally for months thereafter When the infection is due to *S schottmuelleri* 20 per cent of the patients show positive fecal cultures 3 months after the disease The proportion of patients with positive cultures begins to drop off rather rapidly after this to reach a level as low as 5 per cent at the end of the fourth month though a few become chronic carriers

CARRIERS

In some instances the salmonella may establish themselves in the tissue of the host to produce a more or less permanent carrier state after the recovery from an acute infection This is most apt to occur following typhoid fever where about 3 per cent of the cases are found to be excreting *S typhosa* in their stools over a year after recovery from the disease In this instance, typhoid bacilli are present in the gallbladder or, less frequently in the kidney tissue where they multiply and reach the feces or urine sporadically

The carrier state in human beings is observed less frequently with *S paratyphi* and *S schottmuelleri* than with *S typhosa* and its duration is much shorter In a study of a large number of healthy carriers (Seligman

et al 1946) 28 different Salmonella types were isolated with *S schottmuelleri S typhimurium S oranienburg S montevideo S newport S panama* and *S anatum* being the most common Many of these carriers were contacts of cases and had exhibited no clinical symptoms of infection

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tion it is often difficult to assess the results, since general conditions of sanitation began to improve both in military and civil practice at about the same time that vaccination was introduced In consequence, the use of the comparative incidence of typhoid fever, during consecutive periods of years as an indication of the efficacy of vaccination is invalid unless the sanitary conditions are known to be identical in these successive periods For this reason a strictly controlled field trial was organized recently in Yugoslavia by the World Health Organization and it was shown that a phenolized vaccine containing O but not Vi antigen was effective in reducing the rate of infection thus demonstrating the effectiveness of immunization but raising the question of the role of the Vi antigen in protection against typhoid fever (Cvjetanovic 1957) In another study by Callender and Luippold (1943) in which a standard vaccine had been used for the immunization of a group of men who subsequently were exposed to a common contaminated water supply the incidence of typhoid fever among the nonimmunized group was 7 per cent as compared with 1 1 per cent in the vaccinated group These results suggest that the protection conferred by vaccination is only relative since cases did occur in immunized groups Since the infecting dose may be very large in some instances the immune mechanism may be overwhelmed thus explaining the failure of the vaccine to give complete protection under all conditions

The vaccine commonly used consists of a *saline suspension of typhoid organisms in the* smooth phase killed by heating for 1 hour at 56° C The suspension is standardized to contain 1 billion organisms per ml Tricresol or an organic mercurial is added as a preservative

Grinnel (1932) studied the immunizing potency of the various dissociants of the typhoid bacillus and found that vaccines prepared from rough strains had little value This was confirmed by the findings (Felton and Wakeman 1937) that the O somatic antigens which are lost in the S → R dissociation are the essential immunizing constituents of salmonella Recently recognition that the Vi antigen is destroyed by heating has led to the use of an alcohol inactivated vaccine in which the Vi antigen is preserved However, as mentioned above it has not yet been demonstrated that the Vi antigen plays an essential role in protection

Since the O antigens can now be prepared in relatively pure form, they have been used in experimental immunization in man In very small amounts, they produce somewhat higher circulating antibody titers than the equivalent quantity of whole bacterial vaccines with less unpleasant toxic reactions (Morgan 1945)

During periods when sanitation breaks down a wider range of protection may be desired and typhoid vaccine is often supplemented by the addition of suspensions of heat killed *S paratyphi* and *S schottmuelleri* to form T A B vaccine In certain geographic areas this vaccine includes *S hirschfeldii* (T A B C vaccine) These vaccines are given in the form of 3 injections of 0 5 to 1 0 ml subcutaneously and reinoculation with 0 5 ml every 3 years The administration of 0 1 ml intracutaneously as a booster dose produces less fever and undesirable reactions yet it appears to give adequate antibody titers It has been suggested that the intracutaneous route might be used also for primary immunization Administration of vaccines by the oral route has not been shown to elicit an adequate antibody response

The value of antityphoid immune serum used prophylactically in individuals known to have been exposed to typhoid fever has not yet been established

DIAGNOSIS

The diagnosis of Salmonella infection depends upon isolation and identification of the causative agent (e g from blood feces or urine) or upon the demonstration of a rise in titer of specific circulating antibodies for a given organism

Isolation of the organism from the circulating blood is the most conclusive method of bacteriologic diagnosis It is most likely to be successful in the earliest phases of the disease and in the enteric fever or septicemic types of clinical disease In typhoid fever positive blood cultures are obtained in 80 to 90 per cent of cases during the first week (Fig 38) Blood cultures are usually negative in salmonella gastro enteritis

Organisms may be isolated from the feces

in typhoid fever or in salmonella gastro-enteritis during the acute stage of the disease and for a variable period thereafter In typhoid fever the number of positive fecal cultures may increase during the second week Organisms may also be isolated from the vomitus or from the contaminated food ingested in gastro-enteritis In *S typhosa* infection as many as 25 per cent of the cases have positive urine cultures in the later stages of the disease Salmonella may be found in the urine of patients with the septicemic type of infection when the organisms have localized in the kidney

It has been found that several different species of salmonella may be isolated from the stools of patients involved in a single outbreak of salmonellosis and that 2 or 3 species may be found in the same patient (Saphra and Winter 1957)

Isolation of salmonella from the feces presents a special problem because of the huge number of normal fecal organisms (It is important to culture the fecal specimen as soon as possible to prevent overgrowth by the normal fecal flora a direct rectal swab may be a useful adjunct in obtaining a fresh specimen) A variety of materials have been added to special media to aid in the isolation of intestinal pathogens They fall into several categories (1) agents which inhibit the growth of normal intestinal bacteria e g dyes like brilliant green selenium salts and bile salts (2) agents which favor the growth of salmonella over the coliform organisms e g sodium tetrathionate and sodium citrate (3) substances which give to salmonella colonies distinguishing characteristics e g lactose with necessary dye indicators to color the fermenting colonies of the coliform group sulfite which is reduced to sulfide by many salmonella and gives a black color to the colony in the presence of iron salts

The fresh stool specimen should be streaked immediately on a selective medium e g SS agar or sodium desoxycholate citrate agar and on a nonselective medium such as EMB or Endo The nonselective medium is included to pick up the rare species of *Salmonella* that do not grow well on the selective media For use in the isolation of *S typhosa* the bismuth sulfite selective medium is of particular value At the same time the specimen should be inoculated into an enrichment medium which allows the pathogens to multiply and inhibits *E coli* e g brilliant green or tetrathionate broth After incubation for from 12 to 24 hours this enrichment culture is streaked on selective and nonselective agar media Suspected colonies are subcultured and identified by biochemical reactions and by agglutination tests with specific absorbed sera Further species identification of salmonella can be obtained by sending the culture to one of the Salmonella Centers

Serologic Diagnosis

Agglutinins for the causative organisms usually can be demonstrated in the circulating blood of patients from 1 to 2 weeks after the onset of typhoid and enteric fever septicemia or gastro-enteritis of the more severe types In mild diarrheas the agglutination reaction may not become positive until after the symptoms have subsided or in some instances it may remain negative Tests should be carried out for both O and H agglutinins but O agglutinins appear to be of more significance since some cases fail to develop H agglutinins Moreover O agglutinins are of more diagnostic value because they disappear more rapidly than H agglutinins following vaccination Finally O antigen relationships among the various salmonella broaden the range of the test for the initial detection of infection

The antigens used in the agglutination test should be so composed as to give an adequate coverage for all of the principal *Salmonella* groups occurring in the particular geographic area e g O antigens of groups B C and D and A where indicated After this preliminary screening further testing can be carried out to give definite diagnosis of the species involved In the United States *S typhosa* *S schottmuelleri* and *S choleraesuis* are commonly used for screening agglutination tests It is not necessary to include a Vi antigen for the diagnosis of typhoid fever since Vi agglutinins rarely appear in the blood of patients in the absence of H or O agglutinins Also Vi agglutinins may not be detectable in the serum of patients known to have typhoid fever The detection of agglutinins in the feces of patients (Harrison and Banward 1947) has been suggested as a diagnostic method

In the Widal or agglutination test the dem

onstration of a rising titer of specific agglutinins is accepted as definite evidence of infection with the particular *Salmonella* strain. However, if only a single specimen is available, an O agglutinin titer of more than 1:50 during the first 10 days of illness is considered strong presumptive evidence if the patient has not been vaccinated within 2 years. In a patient with a history of previous inoculation with typhoid vaccine, the fact that H agglutinins tend to persist for a number of years following immunization, while O agglutinins fall in 6 months and usually disappear in about a year, makes the O agglutinin titer more valuable in diagnosis. The common concept that such agglutinins will rise in titer during any febrile illness due to a nonspecific anamnestic reaction has been found to be erroneous (Koomen and Morgan, 1954). However, several other factors should be considered in the interpretation of a single agglutination titer. The level of agglutinins in the serum of normal persons in the particular geographic area is important, and in all instances the stage of the disease at which the serum specimen was taken should be noted. Since antigen preparations differ in their sensitivity to the agglutinating action of sera, the activity of each new antigen should be evaluated with a standard serum. The test for O agglutinins can be made much more sensitive by adsorbing O antigens to the surface of human type O erythrocytes. These red cells, when added to serum containing the anti-O antibodies, agglutinate to high titer (Neter et al., 1956).

TREATMENT

The treatment formerly used in typhoid fever or other salmonella infections was mainly supportive and aimed at maintaining the fluid balance and the nutritional state of the patient. More recently, several specific therapeutic agents have been employed. The sulfonamide drugs apparently have had some beneficial effect on certain salmonella infections, but their use in typhoid fever has been disappointing. Combined therapy using sulfonamides with larger than ordinary doses of penicillin has given some evidence of therapeutic value. Streptomycin has had a limited trial in typhoid fever and in other salmonella infections without any clearly beneficial results, in spite of the fact that the organisms are sensitive to the action of the drug in vitro (Keefer, 1946). Streptomycin given orally markedly reduces the number of typhoid bacilli as well as of coliform organisms in the stools. However, the bacteria reappear when the therapy is discontinued.

Chloramphenicol is effective in the treatment of typhoid fever (McDermott, Knight and Ruiz Sanchez, 1949), though patients do not become afebrile until about the fourth day after treatment is started and may become carriers in spite of adequate therapy. However, chloramphenicol therapy of other salmonella infections has been less successful.

EPIDEMIOLOGY

The source of all salmonella infection is the reservoir of organisms living in the tissues of human beings or animals. Infection occurs through food, milk or water contaminated with infected feces or urine or by the actual ingestion of the infected animal tissues. The hosts which harbor the organisms may be clinically recognized cases or sick animals, subclinical cases or carriers. The two latter groups are the most important, since they are usually unrecognized. Infection with most salmonella apparently requires the ingestion of large numbers of organisms, but in the case of *S. typhosa* relatively few bacilli are sufficient to cause typhoid fever. This difference in infectivity probably accounts for the fact that lightly contaminated material like water or shellfish can be the source of typhoid infection, while most other salmonella infections are caused by heavily contaminated food in which the organisms often multiply before ingestion.

Water may be contaminated with infected feces by cross connections between water and sewage pipes, seepage of surface water into wells, or surface contamination of shallow wells. Epidemic outbreaks of typhoid fever involving entire communities may follow a breakdown of the water purification system during a flood, for example. Outbreaks involving fewer individuals may be caused by contaminated milk not pasteurized or, more rarely, contaminated after pasteurization. In these instances, the distribution of cases parallels that of the delivery of the milk. Oysters and shellfish may also cause smaller outbreaks

when taken from water contaminated with sewage

Endemic typhoid fever and infections with other salmonella are now much more common than epidemic typhoid fever in parts of the world where sanitary measures are applied to protect water and food supplies In these instances infection usually is due to contamination of food by a human being or an animal excreting the organisms or to the ingestion of the improperly cooked meat of an infected animal or of foods containing contaminated egg products In some instances flies carry infected excreta to the food or even become infected themselves with the bacilli and deposit them on the food Milk and dairy products such as cream custard ice cream and cheese have been involved in the spread of typhoid fever and other *Salmonella* infections The bacilli can grow in foodstuffs at warm temperatures without producing any detectable changes in appearance or taste to reveal their presence Among foods prepared from tissues of animals infected with *Salmonella* bacilli are meat products like sausage and luncheon meats which are often insufficiently cooked Eggs are often implicated and positive salmonella cultures have been obtained in as many as 30 per cent of a series of preparations of powdered eggs most of which contained *S pullorum* although *S oranienburg* *S typhimurium* and *S montevideo* were also found Ducks are known to harbor *S typhimurium* and *S enteritidis* which have been found in their eggs

Less than 5 per cent of recognized clinical cases of typhoid fever become chronic carriers and continue to excrete *S typhosa* in their stools and in some instances in their urine for years They constitute the main source of infection in this disease although ambulant cases also spread the organisms Since over 90 per cent of typhoid carriers show Vi agglutinins in their serum this provides a valuable screening test for their detection Once proved to shed bacilli in their urine or feces these persons must be isolated and kept under medical supervision until rid of the carrier state by procedures such as cholecystectomy or drug therapy Disappearance of Vi agglutinins suggests that the carrier state has been eradicated but this is proved only by repeated negative fecal and urine cultures As the incidence of typhoid decreases the number of carriers is reduced sharply thus drying up the reservoir of infection

Chronic carriers who excrete organisms for over a year are found much less often with *S paratyphi* and *S schottmuelleri* and even more rarely with other organisms such as *S typhimurium* With other salmonella the most important sources of infection are the temporary convalescent carriers who may continue to excrete the bacilli for from 4 to 16 weeks after their illness and the ambulant subclinical cases who excrete the organisms in their stools In any outbreak there may be a large number of these apparently normal contacts who are excreting the bacilli and therefore spreading the infection

CONTROL MEASURES

The control of *Salmonella* infections is directed at (1) the elimination of the sources of infection (2) the detection and the elimination of the modes and the vehicles of transmission and (3) measures to increase the resistance of the susceptible host The first two aspects of control are the most important and usually are relied on entirely except under circumstances in which control of the environmental factors become more difficult as in time of war when the third aspect may assume a more critical role

In the case of typhoid fever elimination of sources of infection is largely a question of detection and supervision of the chronic human carrier This measure is also valuable in the prevention of the spread of other salmonella infections In all instances the infected patient must be recognized isolated and his excreta carefully disposed of Bacteriologic examinations should be made of the stools in all cases of diarrhea and all salmonella infections should be reported The detection and the isolation of the ambulant cases in an outbreak is a valuable adjunct in control when this procedure is feasible Ideally any individual yielding positive cultures of feces or urine should be isolated until several successive negative cultures have been obtained

Adequate inspection of animals slaughtered for meat is of value in limiting the consumption of meat from sick animals However if the disease is not active the detection of infected animals may be very difficult Inspec

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The Shigella and Bacillary Dysentery

Organisms belonging to the genus *Shigella* (a member of the family *Enterobacteriaceae*) are nonencapsulated nonsporulating nonmotile gram negative rods approximately twice as long as broad They are facultative anaerobes growing best under aerobic conditions Different members ferment various carbohydrates with the production of acid but (with few exceptions) no gas All ferment glucose some ferment mannitol At least one species ferments lactose but only after 2 or 3 days incubation None attacks salicin or adonitol They fail to utilize citrate to liquefy gelatin to decompose urea and to produce hydrogen sulfide or acetyl methyl carbinol Many species share antigens in common with members of other genera of the family *Enterobacteriaceae* in addition however they have specific antigens by which they can be recognized Their natural habitat is restricted to the gastro intestinal tract of primates in contrast with the more ubiquitous distribution of the members of the genera *Salmonella* and *Escherichia* The various species of *Shigella* are pathogenic for man and represent the etiologic agents of bacillary dysentery hence the common name of dysentery bacilli

HISTORY

The clinical entity of bloody or mucous diarrhea accompanied by straining and tenesmus was recognized by ancient authors as long ago as the 4th century B C Herodotus ascribes the defeat of the Persian Army in 380 B C in part to dysentery The clinical and epidemiologic descriptions furnished by Hippocrates in the same century suggest that the disease was well known in Greece and that the bulk of it was probably of bacillary rather than amebic origin Until the recent advent of effective sanitation the conditions associated with military operations have been particularly conducive to the spread of enteric disease and it is not surprising that outbreaks of dysentery have been a frequent accompaniment of military campaigns In recent years the Gallipoli campaign of World War I the operations in the Mediterranean and the Pacific Theaters of World War II and the experiences in prisoner of war camps during the Korean War have furnished invaluable clinical laboratory and epidemiologic data to students of the disease In civil life jails and asylums have long been notorious for outbreaks of dysentery over crowding poor sanitary facilities and low standards of personal hygiene appear to have been the common factors

In spite of the many excellent clinical accounts of dysentery which have been published since the time of Herodotus it was not until the closing years of the 19th century that the bacillary and the amebic varieties were separated on clinical epidemiologic and etiologic grounds The latter disease occurs sporadically rather than in epidemics pursues a chronic rather than an acute course is frequently complicated by heaptic abscese if specific therapy is not instituted promptly and presents the typical pathologic findings of hallow undermining ulcers in the large bowel

tion and supervision of other animal food products also serve to reduce contamination

Spread of the infection can be halted by methods directed at the vehicles and the modes of transmission Modern sanitation methods such as proper sewage disposal se lection of unpolluted sources of water supply with its subsequent filtration and chlorination and the pasteurization of milk have been largely responsible for the dramatic decrease in the incidence of typhoid fever

The *contamination* of *food* may be pre vented by the exclusion as food handlers of persons with diarrheas and by the elimination of rodents and flies from premises where food is prepared Careful handling of food after preparation adequate refrigeration of un cooked foods adequate cooking of meats and of eggs and egg products all serve to reduce *Salmonella* infection

Active immunization with typhoid and *Salmonella* vaccines should be regarded as an emergency measure to be used under condi tions of great exposure for example among the staffs of contagious disease hospitals, among soldiers or travelers in countries where the diseases are still endemic due to lack of sanitary developments or when sanitation breaks down as in catastrophes or periods of war Vaccination is a poor substitute for sani tary control since there is no evidence that it alone can stamp out intestinal diseases though it seems to reduce the incidence and the fatal ity of *Salmonella* infections Cases do occur among vaccinated individuals (Syverton *et al* 1946) especially when the infective dose is large In the prisoner of war camps in World War II vaccination apparently was effective in aiding in the control of disease until the camps became severely overcrowded Prophy lactic vaccination during an epidemic is of value mainly in preventing secondary cases among contacts of infected individuals If the outbreak is explo ive as in the case of a water borne epidemic of typhoid fever where the source can be discovered and controlled early, immunization of the entire community is not necessary, since it can have no effect on indi viduals in the incubation period of the disease and is unnecessary for those who have escaped infection with the exception of those in direct contact with the patients (Topley, 1938)

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teristically slow and is not apparent for several days In the case of *Sh sonnei* at least this property is due to the development of lactose-fermenting papillae on the original lactose negative colonies grown out on primary isolation (Glynn and Starkey 1939) With the exception of these species the members of the genus *Shigella* resemble the other enteric pathogens in their inability to ferment lactose Two strains of *Sh flexneri* 6 form exceptions to the rule that dysentery bacilli are anaerogenic The so-called Newcastle strain may produce a small quantity of gas in both glucose and dulcitol while the closely related Manchester strain may produce gas in mannitol as well A useful point in the differentiation of *Sh dysenteriae* 1 and 2 is the ability of the latter to produce indole as compared with the failure of the former to do so Of the less common types of *Sh dysenteriae* 5 and 7 produce indole while 3 4 and 6 do not While these various biochemical reactions are of considerable usefulness in the tentative differentiation and identification of pecies and types the final identification of the various *Shigella* dep nds upon the study of the antigenic structure

The antigenic pattern of the *Shigella* is complex Most species tend to be antigenically heterogeneous some much more so than others Serologic overlapping between different spe ies occurs due to the presence of common group antigens Furthermore certain of these group antigens are found in other enteric bacilli The fact that some strains of *Shigella* may be rendered more agglutinable by boiling or other processes suggests that in addition to group and type specific antigens these organisms *may contain* K *(or envelope) antigens* as well An additional source of trouble is the occasional instance in which there is a lack of correlation between serologic and biochemical characteristics In such a case antigenic structure usually is accepted as the final criterion for the purposes of classification

Sh dysenteriae 1 (originally isolated by Shiga) and 2 (originally isolated by Schmitz) are antigenically homogeneous That is an antiserum prepared against a strain of a given type will agglutinate all other strains of the same type to approximately the same titer but will not agglutinate strains belonging to other types or species *Sh dysenteriae* 3 4 5 6 and 7 comprising the so-called Large-Sachs group all possess type- pecific antigens by which they may be identified Some sharing of group antigens with certain coliform organisms has been reported and the fact that boiled cultures usually produce stronger agglutination reactions suggests the presence of K antigens

Sh flexneri presents a complicated picture which has given rise to several confusing systems of classification and terminology Madsen (1949) reviewed the work of earlier investigators and reported the results of his own extended observations Six type specific antigens (comprising types 1 to 6) have been recognized by means of absorbed sera In addition to the type-specific antigen limited to the type in question each type contains 1 or more group antigens which may be present in other types as well At least 4 of these group antigens responsible for the numerous cross reactions characteristic of this group have been identified They permit the division of the individual types comprising this group into subtypes In the case of 2 well known strains (X and Y) no type-specific antigen has been identified Therefore these strains are usually considered to be degraded variants which have lost the type specific antigen

The 11 types belonging to *Sh boydii* present a simpler antigenic pattern in that no significant group antigen or antigens have been recognized In his pioneer work Boyd (1940) recognized 6 specific types on the basis of distinctive type antigens while a 7th type was identified by several independent investigators during World War II Four additional serotypes have been recognized more recently (Edwards and Ewing 1955) The ab ence of *group antigens largely eliminates confusing* cross reactions and the typing of the individual members of this group can be carried out with unabsorbed sera

Sh sonnei is essentially antigenically homogeneous although the regular occurrence of a phase variation characterized by the formation of smooth and rough forms of the organism gives rise to some confusion Both the S and the R forms are characterized by th possession of serologically distinct antigens which can be demonstrated by means of cross absorption tests In usual laboratory practice typing sera are employed which contain antibodies against both antigens

and an intestinal exudate which is mononuclear in character The causative agent, *Entamoeba histolytica* probably was discovered as early as 1859, and by 1890 it was firmly established as one of the etiologic agents of the disease syndrome

The first successful attempt to incriminate a specific bacterial agent as a cause of dysentery resulted from the work of Shiga in connection with an outbreak of dysentery in Japan in 1896 He isolated the organism now known as *Sh dysenteriae* type 1, from the feces and the intestinal walls of patients suffering from clinical dysentery He isolated the organism during the acute phase of the disease with considerable frequency but only rarely during convalescence and never from healthy individuals Further proof was furnished by the finding of specific agglutinins against the organism in the blood of patients suffering convalescing or recently recovered from the disease but not in the blood of healthy individuals (Shiga 1898) Two years later the first type of *Sh flexneri* was isolated by Flexner in the Philippines in similar fashion from the feces of patients suffering from dysentery The first adequate description of *Sh sonnei* was given by Sonne of Denmark in 1915 *Sh dysenteriae* type 2, was isolated by Schmitz in a Roumanian prisoner-of war camp during an outbreak of dysentery in 1917 Boyd isolated the first strain of *Sh boydii* in India about 1930 *Sh dysenteriae* types 3 through 7 represent the so called Large Sachs group of dysentery bacilli Although the existence of such a group was suspected as early as 1919 much of our information concerning them comes from the more recent work of Sachs and others in India

MORPHOLOGIC AND BIOCHEMICAL CHARACTERISTICS

Members of the *Shigella* group are slender gram negative rods approximately 1 μ by 0 5 μ and as such bear close morphologic resemblance to other enteric and related bacteria On primary isolation they may be so short as to appear coccobacillary in form They are nonmotile and nonsporulating and no true capsule has been demonstrated

These organisms are usually classed as aerobes (because at least some members possess a cytochrome system) and facultative anaerobes They grow best at a temperature of approximately 37° C Their metabolic requirements, which have not been worked out completely appear to be relatively simple, and the ordinary laboratory media will support their growth Their ability to grow in the presence of various bile salts in contrast with some strains of coliform bacilli is of practical importance in cultural procedures for their isolation from the gastro-intestinal tract They do not liquefy gelatin nor do they produce hydrogen sulfide, they are unable to utilize citrate All members of this group fail to produce acetyl methyl carbinol but are able to reduce nitrates to nitrites Some species produce indole Evidence has been presented to suggest that in the case of *Sh flexneri* 3 at least the tricarboxylic acid cycle may function as the pathway of terminal respiration (Pan et al , 1957)

As in the case of other nonsporulating organisms, their resistance to physical and chemical agents is not remarkable A temperature of 55° C sustained for 1 hour will kill them as will exposure to 1 per cent phenol for 30 minutes However they may survive in sea water for at least 3 days and for a considerably longer period in the dry state particularly if they are kept in the dark Under natural conditions in the stool their survival period appears to be short presumably due to bacteriophage action or because of their sensitivity to the acidity produced by the growth of other organisms Thus it is important to culture stool samples as promptly as possible

Individual species of *Shigella* ferment a variable number of carbohydrates with the production of acid but with two exceptions no gas Although some variation in this ability occurs among strains of a given type or species the fermentation reactions are sufficiently constant and characteristic to be of use in their identification and classification All ferment glucose and none salicin, adonitol or inositol On the basis of their ability to ferment mannitol they may be divided into 2 primary groups (1) *Sh sonnei Sh boydii* and *Sh flexneri* (with the exception of a few strains belonging to type 6) ferment mannitol (2) in the nonmannitol fermenting group fall all 7 types of *Sh dysenteriae* Lactose is fermented by *Sh sonnei* and rare strains of *Sh dysenteriae* 1, however the fermentation is charac

dysentery in man associated with this specific organism is uncertain There is no convincing evidence that other species of *Shigella* produce similar toxins

VARIATION

Arkwright (1921) described smooth and rough forms of *Sh dysenteriae* The observations had been confirmed and extended to other members of the genus by Waaler (1935) and Boyd (1938) The S to R variation resembles closely that described for other gram negative organisms It is associated with a change in colony formation and with the loss of the so-called endotoxin the toxic factor associated with the somatic antigen At the same time the type specific antigen disappears partially or completely Strains isolated from human cases of clinical dysentery are usually in the smooth phase The ability of rough forms to cause clinical disease remains undetermined

Among the *Shigella* the occurrence of phase 1 and phase 2 forms of *Sh sonnei* corresponding to smooth and rough forms has been described already It may be mentioned that Wheeler and Mickle (1945) felt that both these variants were essentially smooth in character although they might be distinguished by their characteristic colonial morphology and antigenic analysis However Kauffmann (1951) feels that this variation is a true S to R one In addition old laboratory strains frequently show a third colonial variant which may be regarded as an extreme R form Boyd (1938) has described an unusual type group variation among strains of *Sh flexneri* Two types of colonies were noted upon subculture of a given strain The first contained a type specific and a group-specific antigen while the second contained only the latter although it did give rise to a colony which still retained some of the characteristics associated with smooth strains Once the type specific antigen was lost there was no tendency to regain it Freshly isolated strains usually contained both the type specific and the group specific antigens

BACTERIOPHAGE

The gram negative bacteria inhabiting the gastro intestinal tract of man are usually susceptible to bacteriophage action as was first shown by d Herelle and in keeping with this specific phages effective against most members of the genus *Shigella* have been isolated In the case of *Sh flexneri* Burnet and McKie (1930) have shown that the various serologic types of this organism exhibit characteristic differences in their phage sensitivity Antigenically similar strains show practically identical reactions toward the series of phages tested Similar observations have been made in the case of *Sh sonnei* by Miller and Goebel (1949) and by Hammarstrom (1949) who was able to divide 1,834 strains of this organism into 68 types and subtypes by means of bacteriophage susceptibility In spite of its potential value for epidemiologic studies bacteriophage typing has yet to prove its usefulness in the identification of types and strain of dysentery bacilli

NATURAL HABITAT AND RANGE OF PATHOGENICITY

The natural habitat of the *Shigella* is the gut of higher primates notably man Other mammals such as dogs have rarely been found to be infected The naturally occurring disease is limited to man and perhaps apes and monkeys kept in captivity and these are the only species that develop recognizable clinical manifestations following the oral introduction of the specific organisms Of course contamination of food and water by human fecal discharges derived from carriers of these organisms may lead to the isolation of dysentery bacilli from these sources

CLASSIFICATION

As is common with most genera making up the family *Enterobacteriaceae* the classification of the *Shigella* is neither easy nor entirely satisfactory The difficulties caused by the different nomenclatures used by American British and Continental workers is illustrated and discussed in the monograph by Kauffmann (1954) The most practical criteria of classification at present appear to be a combination of biochemical and antigenic characteristics Assuming the relative stability of serologic and biochemical characteristics of a given organism a useful classification is that proposed by the Shigella Commission of the International Enterobacteriaceae Subcommittee (1954) The following classifica

The chemical nature of the *somatic antigens* of the *Shigella* group has been studied most fully in *Sh dysenteriae* by W J T Morgan et al (1949) and in various members of the *Sh flexneri* group by Goebel and his co workers (1945) and by Cluff (1954) The former used diethyleneglycol for the extraction of the organisms while the latter two employed aqueous pyridine Although the toxic and antigenic properties of the various components isolated varied with the method of extraction the fractions themselves were of the same general nature The somatic antigen *appeared to consist of a protein polysaccharide* phospholipid complex thus resembling the classic Boivin antigen In general the carbohydrate was present in the form of a non toxic polysaccharide which acted as a haptene to confer serologic specificity upon the smooth organism this material was not found in the rough variant The lipid fraction was non toxic and nonantigenic The protein fraction was represented by a nonantigenic conjugated protein which in the case of *Sh dysenteriae* 1 was identical with that present in *S typhi* The polysaccharide conjugated protein complex derived from the former organism is highly antigenic and apparently represents the simplest unit which will act as a complete antigen In their work with *Sh flexneri* 3 Tal and Goebel (1950) have presented evidence suggesting that the toxic factor of the somatic antigen is an entity distinct from the known *protein lipid and carbohydrate components of* the antigen complex

TOXINS

The complete somatic antigens of the *Shigella* are highly antigenic relatively heat stable and show pharmacologic and physiologic properties resembling those of the analogous antigens of other gram negative bacilli As such they are usually described as heat stable endotoxins When injected parenterally into rabbits mice or guinea pigs they cause diarrhea weight loss inflammation and even hemorrhage and necrosis of the gastro intestinal tract These pathologic lesions closely resemble those found in cases of clinical dysentery in man and it would seem reasonable to suppose that the characteristic signs and symptoms of the clinical disease in man were due in part at least to the release of *endotoxin* (*i e* the toxic component of the somatic antigen) in the gut However, this theory fails to explain the inability of coli form bacilli which possess a closely related toxic component, to cause acute gastro enteritis in adults As compared with any of the true exotoxins the relative toxicity of even the more purified preparations of somatic antigen or endotoxin is not great

In common with the other members of the genus *Shigella* the smooth forms of *Sh dysenteriae* contain a heat stable endotoxin which appears to be identical with the somatic antigen *In addition both smooth and rough* forms of this organism produce a specific thermolabile toxin which is fully antigenic in and highly toxic for mice rabbits and guinea pigs Since this material may be separated easily from the cell bodies it is usually referred to as Shiga exotoxin, although there is no evidence that it is actually excreted by the living cells In general methods favoring the autolysis of cells give the highest yield Several methods for the rapid production of this toxin have been described (Dubos and Geiger 1946) as well as the role of iron and related metals (van Heyningen 1955) and of carbohydrate metabolism and pH (Engley 1952) on the production of this substance It is protein in nature and of an approximate molecular weight of 75 000 It may be concentrated and purified by selective precipitation This purified material is relatively heat *labile Injected into rabbits* mice or guinea pigs it causes paralysis of the limbs diarrhea and death and because of the first of these properties it is sometimes referred to as Shiga neurotoxin It is highly antigenic and stimulates the production of a specific antitoxin which neutralizes the lethal effect of the toxin combining with it *according to the* law of multiple proportions It may be detoxified by formalin at an alkaline pH (Farrell and Ferguson 1943) or by ultraviolet radiation (Branham and Habel 1946) and the resulting toxoid is still antigenic Other means of detoxifying these toxins have been reported as has been reviewed by *Engley (1952)* The injection of toxoid into human volunteers has produced local and systemic reactions comparable with those following vaccination against typhoid fever (Farrell 1944) The relationship to the pathogenesis of clinical

serious form of the disease than do other types or species

IMMUNITY

The mechanism of spontaneous recovery from bacillary dysentery is not understood and attempts to elucidate the role played by various immune mechanisms have been much handicapped by the absence of a suitable laboratory animal in which a gastro-enteritis may be established by the natural route of infection Although humoral antibodies appear in response to infection there is no evidence that they affect the recovery process directly nor is there a correlation between their presence or absence and the occurrence of relapses and second attacks Coproantibody has been detected in the intestinal discharges of individuals suffering from bacillary dysentery (Barksdale and Ghoda 1951), and it is possible that this type of antibody may have a more significant relationship with the mechanisms of recovery and immunity in this disease The local tissue response may be a factor in this process the exudate in bacillary dysentery is rich in polymorphonuclear leukocytes and it is plausible that the presence of coproantibodies against the somatic antigen may enhance the opsonization and subsequent phagocytosis of the infecting organisms The use of the indirect bacterial hemoagglutination or hemolysis technics for the demonstration of small amounts of antibody may be the means whereby the role of these antibodies may be determined (Neter 1956) As in other enteric infections there is no evidence that bacteriophage plays a decisive role

Although relapses and second attacks of bacillary dysentery do occur it is common experience in the tropics that individuals settled in areas where the disease is endemic tend to become immune to frank clinical attacks The nature of this clinical immunity is not clear and careful bacteriologic and immunologic studies will be necessary to determine if it is species specific only or perhaps broader giving some protection against other members of the genus as well

Although the injection of killed dysentery bacilli into human or animal subjects stimulates the production of antibodies which confer considerable protection upon mice against the intraperitoneal inoculation of the homologous organism the results of controlled experiments in which human volunteers were first vaccinated and then challenged by the oral route with the same type have shown no evidence of significant protection against infection (Shaugnessy et al 1946) Field trials (Hardy et al 1948 Higgins et al 1955) have yielded similar results Inasmuch as the disease process is limited to the intestinal mucosa and there is no tendency to invade the bloodstream one would not expect humoral antibodies alone to be effective in the prevention of the disease Studies of the formation of coproantibody following immunization have yielded inconclusive results

DIAGNOSIS

The laboratory diagnosis of bacillary dysentery is made most satisfactorily by the isolation and the identification of the specific causative organism During the acute phase of the disease the organisms are excreted in large numbers in the feces and fresh stool cultures frequently give positive results particularly if the sample contains mucus as the bacteria are found in greatest numbers in this exudate (Boyd 1940) Since dysentery bacilli tend to die out rapidly the stool must be cultured as soon as possible after being voided The suspension of fecal material in a preservative such as buffered glycerol saline solution is of some use if delay is unavoidable The rectal swab technic as first employed by Hardy et al (1942) offers practical advantages in the collection of specimens and their prompt plating on suitable media at the bedside but has the disadvantage of not permitting the microscopic examination of the stool for ova parasites and type of exudate During the acute phase of the disease there is little choice between this method and that of examining freshly passed stools as regards the percentage of positive isolations Sigmoidoscopy with culturing of the actual ulcerations of the intestinal wall under direct observation (Ferris and Fortune 1944) yields the best results and is the method of choice in chronic stages of the disease when the organisms are relatively sparse

Mucus or failing this fecal material is emulsified in saline before streaking At the same time the type of cellular exudate may be determined by examination of a simple

tion follows the compromise scheme pro posed by Cowan (1956) on the basis of the report of this Sub Committee *Sh alka lescens* and *Sh dispar* formerly included in the genus *Shigella* are not discussed in this chapter since it is the opinion of most authori ties that they should be classified in the genus *Escherichia* as representing nonmotile anaero genic members of this group

CLASSIFICATION OF SHIGELLA

SCIENTIFIC NAME		COMMON NAME
Sh dysenteriae	Type 1	Shiga s bacillus
	Type 2	Schmitz s bacillus
	Types 3 7	Large Sachs bacilli
Sh flexneri	Types 1 6 *plus 2* variants	Flexner s bacilli
Sh boydii	Types 1 11	Boyd s bacilli
Sh sonnei		Sonne s bacillus

PATHOGENESIS

Dysentery bacilli usually reach the gastro intestinal tract via the oral portal of entry through the medium of infected fingers food or water In direct contrast with the enteric fevers no bacteremic phase occurs and the organisms remain limited to the duct wall The essential pathologic process is an inflam matory one which always involves the large bowel and occasionally the terminal ileum as well Inflammation of the mucous membrane of the bowel wall is followed by necrosis which in more severe cases goes on to actual ulceration penetrating as deeply as the mus cularis mucosa In contrast with amebic dysen tery the edges of the ulcers remain sharp and undermining does not occur In all but mild cases some degree of hemorrhage takes place The intervening mucosa is inflamed and edematous, and microscopic examination of the bowel wall shows it to be infiltrated with polymorphonuclear cells As the process sub sides granulation tissue replaces the ulcera tive lesions and in severe cases scar tissue may develop

It must be admitted that the mechanisms responsible for these pathologic changes have not been clarified It is believed by some that the pathologic changes follow upon the local irritative action of the heat stable endotoxin released by the autolysis of the bacterial cells which are found in large numbers on the floor of the ulcers and frequently in the inflamed mucosa as well Direct experimental proof of this is lacking although the parenteral injec tion of large amounts of dysentery bacilli autolysates into laboratory animals gives rise to somewhat similar lesions Furthermore there is no clear cut evidence bearing on what fac tors govern the severity of a given infection which may run all the way from an inappar ent affair to a severe rapidly fatal attack of dysentery

The classic clinical picture of bacillary dysentery is dominated by diarrhea abdom inal pain and fever The incubation period is variable but may be as short as 24 hours Abdominal discomfort and cramps (often de scribed as "griping") are the first symptoms and usually come on suddenly They are fol lowed shortly by diarrhea which in all but the milder cases is accompanied by straining and tenesmus The stools are liquid almost from the start large amounts of mucus are passed and in the more severe cases blood as well The diarrhea and the abdominal cramps reflect the acute inflammation of the large bowel straining and tenesmus furnish evidence that the process involves the rectum The fever which accompanies the typical severe case presumably is due to the ab sorption of toxic products from the gut The disease *tends to be self limited and uncom* plicated recovery is the general rule Cases of chronic relapsing dysentery are usually of amebic origin Complications are rare and although some authorities feel that the major ity of cases of 'idiopathic ulcerative colitis' are sequelae of chronic bacillary dysentery a causal relationship between the two remains to be proved A small proportion of recovered patients become chronic carriers of dysentery bacilli for varying periods of time These and even more so individuals with inapparent in fections constitute a major problem in the control of the disease

The clinical severity of a given case of bacillary dysentery is modified by nonspecific factors such as the age and the general con dition of the patient and the opportunities for supportive therapy Some correlation ex ists between the species involved and the severity of clinical manifestations inasmuch as *Sh dysenteriae* 1 in general causes a more

on the part of the bacteriophage preparations employed was obtained

EPIDEMIOLOGY

Bacillary dysentery is traditionally regarded as a disease of the tropics however it has a world wide distribution in actual fact *Sh flexneri* and *Sh sonnei* present a particularly wide distribution *Sh dysenteriae* 1 usually associated with a clinically severe form of the disease has been found in significant numbers only in the Far East in recent years and even here its importance as a causative organism of the disease appears to be on the wane

The source of infection is essentially man The *Shigella* are strict parasites and no natural animal reservoirs have been detected Their natural habitat is the gastro intestinal tract of man Here they may cause a fulminating case of classic dysentery a mild diarrhea or perhaps most frequently an inapparent or subclinical infection Because of this wide variation in severity of clinical symptoms the term Shigellosis is often applied to infections (apparent and inapparent) caused by this group of organisms It is well recognized that the organisms are excreted in large numbers during the acute phase of clinical dysentery It is not so well recognized that the patient with a mild diarrhea or with no symptoms whatsoever may also be acting as an excretor of the organism As the patient recovers from the clinical attack of the disease the organisms tend to disappear concomitantly from the stools although in some instances they may persist during convalescence or even longer The development of a true carrier state is of rare occurrence perhaps due to the fact that dysentery bacilli show no tendency to invade the biliary tract Clinical attack rates are higher in children as compared with adults in the same community Presumably the development of a specific immunity plays some part in this finding

The spread from man to man may take place by a variety of methods The contamination of human fingers with fecal material containing dysentery bacilli is the first step in the direct transfer of organisms from one individual to another (Hutchinson 1956) The contamination of inanimate objects such as toilet seats door handles toys pottery and glassware which are used in common by many people represents an intermediate step in the process Outbreaks occurring in institutions such as asylums or enclosed military groups are usually due to this type of spread as a general rule as standards of personal hygiene improve the incidence of *Shigella* infections diminishes As Watt (1956) has emphasized a high prevalence of these infections in a given community usually is associated with the more or less direct transfer of infected human feces from one individual to another The effect of water availability upon the prevalence of *Shigella* infections has been studied by Hollister (1955) and others In general infection rates were found to be highest in areas where water was least available for personal hygiene

Spread from man to man may occur more indirectly through the medium of contaminated food or water supplies Foodstuffs are usually infected by the dirty fingers of food handlers or by flies which have access to fecal material containing dysentery bacilli Genuine water borne outbreaks have been comparatively rare and usually occur through the medium of faulty plumbing or lack of protection of the source of supply In warm areas where there is usually a large fly population these insects may play a major role in the spread of these organisms if they are permitted to feed indiscriminately upon human feces and food It should be emphasized that the transmission of *Shigella* by these insects is primarily a mechanical one as there is little evidence that multiplication takes place to any extent in the gut of the fly The low standards of environmental sanitation frequently found in the tropics and the presence of a large fly population are important factors in the high prevalence of clinical dysentery in these areas

CONTROL MEASURES

The logical control of bacillary dysentery falls under 4 headings (1) elimination of sources of infection (2) prevention of spread (3) increasing individual resistance (4) chemoprophylaxis Of the 4 the second i e prevention of spread has played through the application of a variety of community and individual measures the dominant role *in re-*

stained smear A predominance of polymorphonuclear cells suggests dysentery of bacterial origin, while a predominance of mononuclear cells favors a colitis due to *Entamoeba histolytica* Swabs are plated out directly As in the case of other enteric infections selected media are of the greatest advantage in primary isolation work

All these contain lactose and an indicator such as neutral red which will detect any fermentation of this sugar Such media permit rapid differentiation between prompt lactose fermenting organisms such as the coliform bacilli and late or nonlactose fermenting bacteria such as the dysentery bacilli It is best to use 2 media one relatively noninhibitory such as MacConkey s agar and the other an inhibitory one such as *Shigella Salmonella* thiosulfate citrate bile agar, which largely suppresses the growth of coliform bacilli as well as that of gram positive organisms Selenite broth and other enrichment media are of limited usefulness in the isolation of *Shigella* Suspicious colonies are fished and inoculated into Kligler s iron agar Those cultures showing an alkaline slant and acid butt without the formation of hydrogen sulfide or other gas are inoculated into carbohydrate broths (lactose glucose mannitol xylose sucrose salicin and dulcitol) for the detection of characteristic fermentation reactions into peptone water for the determination of indole formation into semisolid agar for the determination of motility and on urea agar for the detection of urease activity In connection with fermentation reactions it must be remembered that *Sh sonnei* is a late lactose fermenter which rarely attacks this sugar before 48 hours and usually later

Once an organism has been grouped provisionally by means of these biochemical reactions its final identification should be carried out by the use of specific typing antisera employing the method of slide agglutination

Since as was first shown by Shiga in 1896 agglutinins appear in the bloodstream during the course of the disease it is theoretically possible to make a serologic diagnosis by the demonstration of the formation of specific antibodies against a given organism during the course of illness At best this furnishes a retrospective diagnosis and in actual fact this technic is relatively unsatisfactory since these antibodies develop irregularly and the multiplicity of species and types capable of causing the disease necessitates the setting up of the agglutination test against a large number of bacterial suspensions

SPECIFIC TREATMENT

Antimicrobial therapy with the broad spectrum drugs (tetracyclines and chloramphenicol) has been markedly effective in the treatment of bacillary dysentery As the result of extensive field trials carried out in the prisoner of war camps in Korea Hardy and his associates (1952) demonstrated that the administration of any of these drugs in relatively small amounts over a period as short as 24 hours resulted in the prompt disappearance of the clinical manifestations in nearly all cases This clinical improvement was paralleled during the same time period by a rapid conversion from positive to negative cultures As yet the emergence of drug resistant organisms and the occurrence of relapses have not constituted significant problems This stands in marked contrast with earlier experience with sulfonamide drugs During World War II many of these agents were shown to exert a bacteriostatic effect upon members of the genus *Shigella* in vitro and at first clinical trials with both the soluble compounds (e g sulfadiazine) and the insoluble ones (e g sulfaguanidine) yielded markedly favorable results However, the rapid emergence of drug resistant strains (particularly *Sh flexneri* 3 and *Sh sonnei*) has sharply limited the usefulness of these agents and today the broad spectrum antibiotics mentioned above are the drugs of choice

Serotherapy has been disappointing and today finds little use Convincing proof as to the efficacy of high titer antitoxin against the so-called exotoxin of *Sh dysenteriae* 1 in modifying the course of disease caused by this organism is lacking A polyvalent antiserum has been used in the treatment of infections due to the various types of *Sh flexneri* with little or no success As in the case with other enteric infections the results obtained with bacteriophage have been disappointing The carefully controlled experiments which Boyd and Portnoy (1944) carried out in North Africa during World War II may be cited as an example of a field trial in which no evidence of therapeutic or prophylactic efficacy

groups *Salmonella Shigella Arizona Bethesda Escherichia Klebsiella (Aerogenes Aerobacter)* Providence (29911 of Stuart et al) in Rome September 1953 Internat Bull Bact Nomenclature & Taxonomy *4* 47 94

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ducing the prevalence of the disease Since man represents the only recognized host of the *Shigella* the elimination of these organisms from man should remove the great reservoir of the disease At present this is an unattainable goal, since the detection and the isolation of clinically mild and of inapparent infections represent an insuperable problem at the moment As pointed out previously inapparent infections probably outnumber clinical cases of the disease by a considerable margin These facts limit the extent to which the elimination of the source of infection can be carried (Philbrook et al, 1948) The intermittent shedding of organisms by convalescent patients and healthy carriers presents a further complication Nonetheless rigid precautions should be taken with the excreta of patients suffering from recognized infections and these individuals should not be released from isolation until stool or rectal swab cultures are negative on 3 or more successive days

The spread of organisms from one infected individual to another can be reduced by measures falling into 2 large groups On the individual basis high standards of personal hygiene will do much to prevent the direct spread of these organisms from one individual to another The so called asylum dysentery *found both in the United States and Western* Europe is a case in point Bacillary dysentery is often endemic in large mental asylums and in orphanages where overcrowding and poor sanitary habits of mentally defective youthful inmates gives every opportunity for the transfer of fecal material from one individual to another The thorough washing of hands particularly after defecation and before meals is one of the simplest and most effective methods in reducing this type of spread On a community basis those sanitary measures aimed at providing a safe supply of water milk and food to the community are all important as is the operation of an adequate sewage disposal system As has been demonstrated by Watt and Lindsay (1948) effective fly control is often a potent factor in reducing the prevalence of the disease in communities where both food and human excrement are not protected from these insects

Attempts to increase the resistance of the individual against infection by means of active immunization have not been successful to date The toxicity of available vaccine preparations and the multiplicity of potential etiologic agents are factors which have handicapped trials of this method As yet no significant relationship has been proved between protection again the clinical disease and the presence of humoral or coproantibodies

The efficacy of certain antimicrobial agents in eradicating infections with dysentery bacilli has given hope that their use in chemoprophylaxis may exert a significant effect upon the prevalence of disease During World War II mass chemoprophylaxis with relatively small amounts of sulfadiazine was reported to be effective in checking outbreaks of the disease However the emergence of drug resistant strains soon made this type of chemoprophylaxis ineffective The demonstration of the effectiveness of the tetracycline series of drugs in the treatment of these infections renders it logical to employ these agents in chemoprophylaxis as well Although the reported experience of Higgins et al (1955) in Egypt was not encouraging further field trials under controlled conditions are indicated in order that the potential efficacy of this means of prophylaxis may be evaluated

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Pasteurella

INTRODUCTION

The genus *Pasteurella* (tribe *Pasteurelleae*) named by Trevisan in 1885 to honor Pasteur comprise organisms that produce pasteurellosis (hemorrhagic septicemia) plague pseudotuberculosis and tularemia The 4 species share certain staining (gram negative bipolar), cultural (aerobic facultative carbohydrate fermentation and absence of gas production of milk coagulation and of gelatin liquefaction) and parasitic characteristics But certain other characteristics so divide the present group that it seems likely that eventually the type species *Pasteurella multocida* will stand alone in this genus *Pasteurella multocida* and *P pestis* have different nutritional requirements and their usual parasitism and infection chains are dissimilar Despite its antigenic relationship to *P pestis P pseudotuberculosis* is motile and in some respects seems to be more related to the *Enterobacteriaceae Pasteurella tularense* is separated from the other members by its extreme polymorphism its physiologic and biochemical characteristics nutritional requirements and marked cytotropism

All 4 are animal parasites, and their parasitism on man represents an aberration It is mainly when the infections in animals become acute that the parasites abound in man s environment and then he is included as a host As a general rule they do not adapt and continue their usual latent form in the human host

PASTEURELLOSIS

Pasteurella Multocida (Septica)

These organisms are small gram negative ovoid rods showing bipolar staining with Giemsa or carbol fuchsin They include nonmotile aerobic to facultative anaerobic bacillary forms that require a low oxidation reduction on primary isolation Their fermentation of several carbohydrates without forming gas is characteristic They produce indole in appropriate media frequently H S they do not liquefy gelatin or coagulate milk, and they require nicotinamide and pantothenic acid as accessory growth factors

Pasteurella of this species have been isolated from a wide variety of mammals and birds and have been named accordingly Although there are demonstrable difference between these strains from different sources the essential characteristics are so similar that all are included under the *multocida* or *septica* species They are (1) *P bollingeri* or *bovisepitca* (2) *P avicida* or *aviseptica* (3) *cuniculicida* or *lepiseptica* (4) *Bacterium bovicida* or *Pasteurella bubalseptica* (5) *P vituliseptica* (6) *P muricida* or *Bacillus bipolaris* (7) *P pneumotropica* and (8) *P gallinarum P hemolytica* is a closely related organism that causes pneumonia in sheep and cattle, but because it does not cross agglutinate with *P multocida* it is considered a separate species

The host spectrum is broad The naturally occurring pasteurelloses in fowl rabbits herbivores and rodents seem to be diseases of

devitalization Under conditions of lowered host vitality *P multocida* already being carried by the animals on the mucous membranes becomes pathogenic Once this happens in a herd or a flock the disease is likely to spread rapidly in its destructive septicemic form Because it has been rarely recognized as an infection of man the mechanism of infection is not well known It may be introduced through bite wounds or latent infection in the respiratory tract may be mobilized by injury or coexisting disease

History

Discovered at the dawn of bacteriology these organisms were used extensively in early studies of bacteria and immunity It was found that fowl cholera rabbit septicemia Wildseuche (a fatal disease of deer) and cattle swine and buffalo plague were caused by bacilli which because they stain deeply at the poles were designated *Bacillus bipolaris septicus* Overstressing of the bipolarity at first caused great confusion so that the early descriptions of the organism and the disease it causes are not necessarily of *Pasteurella multocida* as it is defined at the present time Lignieres helped to clarify its bacteriologic position but his zoologic grouping according to animal origin of each strain has been modified to accommodate new knowledge The similarity of 230 strains isolated from reindeer cattle buffaloes sheep pigs cats chickens rabbits and rats was great none were motile and none fermented lactose (Schutze 1929) Bacterial characteristics and similarity of pathologic lesions make it likely that the various types all belong to a single species Bergey s *Manual of Determinative Bacteriology* includes under the name *P multocida* Kitt all typical indole producing nonhemolytic organisms Topley and Wilson prefer *P septica* The innumerable strains composing the group fluctuate easily and continuously in physiologic functions antigenic structure fermentation capacity and pathogenicity They are parasites of domestic and wild animals birds and man They may exist on the mucous membranes with little or no pathogenicity The first bacteriologically proved human infection was reported in 1913 by Brugnatelli

Morphology

P multocida varies from small short oval forms to coccobacilli with convex sides and rounded ends Their length ranges from 0 3 to 1 25 μ their diameters from 0 15 to 0 25 μ and they appear singly in pairs chains or clusters Active healthy organisms from cultures stain easily and diffusely with aniline dyes and are gram negative in animal tissues and fluids the ends of the rod stain more intensely than the central portion does The bipolar staining corresponds to the location of the chromatin bodies or of reserve substances surrounding the chromatin granules Pleomorphism is not common although filamentous granular or barred bacilli without bipolar staining are seen in preparations from rough colonies The organisms do not form spores are not motile and have no flagella The capsules which account for the viscosity of the cultures can be shown in counterstained India ink or Giemsa stained preparations Polysaccharide dyes (Alcian blue 8GS phthalocyanin and monastral blue) tinge the capsular material of certain strains in the peritoneal exudate of infected mice distinctly but not intensely There is evidence that the capsules of different strains differ in composition Capsulated strains are not agglutinable but they can be made so by decapsulation by heating the cultures for 10 minutes at 100° C (Bain 1955)

Cultivation and Biochemical Activities

Moderate growth occurs on ordinary alkaline or neutral media at 37 C Under aerobic conditions *P multocida* will develop through 2 or 3 transfers in hydrolyzed gelatin or amino acid basal media with nicotinamide pantothenic acid and aneurin concentrates (Jordan 1952 Banerji and Mukherjee 1954) Colonial forms can transform from virulent to avirulent strains or in the sequence mucoid to smooth to rough Cystin is an essential amino acid lactic acid and sucrose are adequate carbon sources During metabolism in the presence of glucose hydrogen peroxide is formed catalase and hematin will promote growth (Jordan 1952)

The dissociation pattern is as follows (Carter 1957) (1) Mucoid (M) Probably

a phenotypic characteristic Moist, white to reddish opaque fluffy fluorescent colonies measuring up to 3 mm on serum agar in 24 hours at 37° C cells well capsulated (++), slimy precipitate in acriflavine Hemagglutination type, A Inagglutinable in standard agglutination test Highly pathogenic for mice and rabbits in small doses given intraperitoneally some strains lack a toxic component and require 3 days to cause fatal infections (2) Smooth (S) Fluorescent, diffuse growth Hemagglutination type, B Greenish iridescent colonies measuring 1 to 1 5 mm after 24 hours No flocculation in acriflavine Capsule + Cells remain in suspension Pathogenic for mice and rabbits (3) Smooth (S) Non iridescent Intermediate antigenic type S Capsulated Discrete small colonies Blue colonies noncapsulated Partial flocculation in acriflavine Hemagglutination type D (4) Smooth (S) Antigenic type SR Granular blue No capsule Coarse clumping in acriflavine Hemagglutination type C (5) Rough (R) Colonies measure 1 mm after 24 hours Resemble smooth colonies but are drier and difficult to emulsify Agglutinate specifically No capsule Flocculation in acriflavine Nonpathogenic for mice (6) Dwarf Rare Pinpoint colonies near M colonies

Strains are usually isolated in the M or S phase Few colonies dissociate to S or SR forms most remain stable for 20 or more fortnightly transfers Once dissociation starts it usually continues until R forms arise S phases vary in stability but most become R in varying degrees on repeated subculture All phases cause uniform turbidity in broth R forms a granular sediment They do not liquefy gelatin No visible growth takes place on potatoes or MacConkey s medium containing sodium taurocholate Cultures may have a characteristic musty odor

In general *P multocida* produces acid in 0 2 per cent tryptone (Difco) medium containing glucose saccharose fructose (levulose) sorbitol galactose mannose xylose and mannitol Some ferment arabinose glycerol trehalose maltose and even dulcitol and raffinose Immunologically related strains from different countries have characteristic carbohydrate fermentation patterns (Rosenbusch and Merchant 1939) Strains isolated in some countries do not use mannitol All members reduce nitrates and form ammonia indole and H S in small amounts they give a positive catalase test but a negative methyl red reaction, many reduce methylene blue True *P multocida* does not form hemolysin but does reduce oxyhemoglobin and hemoglobin Some strains pathogenic for sheep and cattle but not for mice or rabbits, identical in hemagglutination and agglutination tests, produce atypical hemolysis on rabbit or horse blood agar These are considered to be *P hemolytica* (Carter, 1956)

Drying sunlight heating above 50° C 0 5 per cent phenol or 0 1 per cent formalin kill *P multocida* in 15 minutes Virulent stock cultures can be preserved on blood agar in sealed tubes or by lyophilization Ten strains tested were destroyed in vitro by penicillin chloramphenicol, tetracyclines polymyxin B erythromycin or carbomycin, but some survived with bacitracin or streptomycin (Gorzynski and Neter 1953)

Antigenic Structure

For several decades attempts have been made to classify *P multocida* by serologic and immunologic methods Direct agglutination tests have distinguished 3 types (Little and Lyon 1943) precipitin and capsular swelling tests, 3 or 4 types (Carter and Byrne 1953) cross protection tests 6 types (Roberts 1947) More direct chemical methods of studying the antigens in cell extracts are now yielding more precise information Type specific capsular polysaccharides have been extracted from cultures heated at 56° C for an hour The different dissociation phases have yielded compounds that varied with respect to nitrogen reducing substances phosphorus and physical properties According to results with the agar gel diffusion technic, the M substance readily soluble in distilled water is a complex substance (Carter and Annau 1953 Carter 1957) rather than pure hyaluronic acid as originally believed The soluble substances isolated from the fluorescent and the intermediate variants are specific and protective for mice The protein antigen shows some similarity to fractions extracted from *P pestis* (Bain 1955) The M substance is less immunogenic None of the antigens studied is toxic for mice The specificity of the capsular antigens was established by absorbing them to human type O erythrocytes and subjecting them to the action of different immune sera in the hemagglutination test

(Carter 1955) Independent of animal or geographic source 4 different serotypes—A to D—have been identified Mucoid cultures lose their mucoid character on repeated transfer they then become smooth and can be typed Markedly mucoid strains fall into type A The serologic type C may be difficult to type because of the absence or early loss of capsular substance The Oudin technic may help to reveal differences in certain rough colonies that cannot be typed in the hemagglutination test (Bain 1954 1955)

Old and heated cultures of pathogenic strains yield soluble substances that produce stupor diarrhea edema and death in laboratory animals the toxic effects probably are due to endotoxins liberated during autolysis

Distribution and Range of Pathogenicity

Pasteurella are commensal or parasitic in certain animals In acute fatal infections they may be isolated from blood organs and exudates In chronic infections their precise relationship to the pulmonary lesions and the abscesses in which they are seen is not entirely clear Pasteurella are found very frequently in the upper air passages and less frequently in the intestinal tract of normal cattle horses swine sheep fowl dogs cats and rats Through passage a commensal strain may acquire marked virulence and then may suddenly become commensal again Members of this species produce hemorrhagic septicemia and exhibit host specific pathogenicity The total range of susceptible species is wide including man rodents herbivores domestic and wild birds and carnivores Strains isolated from human infections are pathogenic for mice rabbits and occasionally for pigeons and produce local abscesses on subcutaneous injection of guinea pigs The strains have never been typed antigenically

Pathogenesis

If the bacilli are highly virulent and the host resistance slight the clinical picture is that of acute or peracute septicemia bacilli multiply rapidly in the tissues causing death within 12 to 36 hours There are high fever cardiac weakness prostration toxemia anorexia and sometimes diarrhea Autopsy findings aside from enormous numbers of bacilli in the blood may be few or there may be blood tinged effusions in the serous and mucous membranes and parenchyma of organs The spleen is not enlarged but the lymph nodes are swollen

When the disease lasts for several days localized inflammation and necrosis induce a variety of clinical pictures Pathologically there are hemorrhagic serous infiltrations fibrinous pneumonia with localized caseating necrotic foci serofibrinous exudation of the pleurae

Pasteurella sometimes play an important secondary role in other infections by invading respiratory mucous membranes damaged by the concomitant infection

Immunity

Individual variations from extreme susceptibility to complete resistance hereditary or acquired immunity and seasonal or other environmental factors apparently govern the course of the disease Although the illuminating observations of Pasteur on the loss of virulence of fowl cholera organisms and on the immunizing value of avirulent cultures have been of fundamental importance in the study of immunity immunization against pasteurellosis is rarely practical It is of concern in areas where livestock losses are heavy The rarity of human infections has made it unnecessary to develop methods of immunizing man against this infection Many attempts have been made to immunize animals (Delpy 1952) Formalized suspensions of strains of high virulence rich in capsular antigen or particularly vaccines in adjuvants have conferred immunity if the antigenic type or types in the epizootic have been selected properly Chick embryo vaccines aggressins filtrates of broth cultures or saponic vaccines have been of little value Avirulent living cultures of adequate antigenicity have protected cattle against infection

Sera of immune animals continue to be used in the hope of salvaging infected poultry flocks and livestock herds The value must be questioned because the immunizing strains are selected according to animal species from which they have been isolated rather than according to serologic types It is of course possible that some types may have a regional distribution and in this case treatment based

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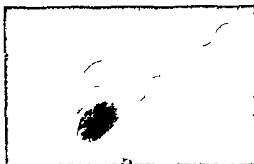


FIG 37 Electron micrograph of P pseudotuberculosis type I (strain 2) U tube cultivation at 22 C flagellated smooth form × 12 000 (Knapp W unpublished)



FIG 38 Electron micrograph of P pseudotuberculosis type I (strain 2) U tube cultivation at 22 C flagellated rough form × 12 000 (Knapp W unpublished)

some physiologic disturbance of the infected tissues is an acute infection provoked Localized infections on the hands which may be limited to phlegmon myositis and abscesses have been complicated by osteomyelitis (Allott et al 1944 Hansmann and Tully, 1945 Reilly and Tournier 1954) It is very difficult to know the true pathway of infection—to know whether these organisms were introduced through the skin or the mucous membranes or whether organisms already present found access through breaks in these surfaces

The sera of patients tested in the course of subacute infections yielded agglutination or complement fixation titers in dilutions of as high as 1 3 200 with the pasteurella isolated from the lesion Allergic skin tests are claimed to be useful in differential diagnosis

CONTROL MEASURES

Human infections probably will continue sporadically because it is improbable that the infection will be eliminated from the widespread animal reservoirs through which it is usually contracted Proper feeding and sanitary management of chicken flocks and hog ranches are more effective and economical than treatment or biologic prophylaxis In countries of primitive sanitation vaccination of cattle buffaloes and hogs still has its advocates

PLAGUE

PASTEURELLA PESTIS

Plague bacilli are large gram negative elongated rods with rounded ends which stain at both poles in the parasitic stage coccus like round filamentous or other pleomorphic forms are common (Figs 37 and 38) These nonmotile organisms grow on media containing bile salts ferment carbohydrates only slightly (saccharose not at all) produce neither indole nor sulfurated hydrogen are milk neutral and require no accessory growth factors

P pestis cau es an infection primarily of wild rodents and rats maintained as a continuous heterogenous infection chain by an insect vector the flea Man becomes a victim of the bubonic form as an aberrant interruption of the rat flea rat sequence or by handling infected wild rodents If the bubonic form becomes generalized and the lungs are secondarily affected man to man infection ensues without intervention by the flea

(Synonyms Bacille de la peste [Yersin] Pestbacillus [Aoyama] *Bacterium pestis* [Lehman and Neumann] *Bacillus pestis* [Migula] *Yersinia pestis* [van Loghem])

HISTORY

The history of plague can be traced back almost uninterruptedly to the 3rd century before the Christian era when Dionysius told of it as a fatal disease in Libya Egypt and Syria The pandemics and the epidemics of history have been examined and interpreted in the works of Simpson G Stricker Hirst (1953) and Pollitzer (1954) For centuries Black Death found a highly susceptible population living in poverty in Europe and took an appalling toll justifying all of its somber aliases and the awe in which it always has

on knowledge of the local types would be expected to succeed

Diagnosis

Painful local inflammation or abscess formation following animal bites or other animal contact deserve careful bacteriologic examination Pasteurellosis arising from sources other than animals has been diagnosed through systematic clinical and bacteriologic examination Neither clinical findings nor anatomic changes are pathognomonic Agar medium with 1 per cent hemolyzed rabbit blood is particularly useful in obtaining single colony growth Microscopic cultural and biochemical examinations and pathogenicity tests on mice guinea pigs rabbits and pigeons supplemented by serologic tests with typing sera will readily differentiate *P multocida* from *P pestis* and *P pseudotuberculosis rodentium*

Chemotherapy

P multocida is very susceptible in vitro to penicillin tetracycline chloramphenicol and polymyxin B but is rather resistant to streptomycin neomycin and is insusceptible to bacitracin Limited observations indicate that chlortetracycline is somewhat more effective than oxytetracycline The clinical response to penicillin has been favorable Oxytetracycline intramuscularly is superior to penicillin in the treatment of fowl cholera continuous administration of sulfamerazine in the mash or drinking water effectively controls the disease when it is begun before infection

Epidemiology and Epizootiology

Since this infection does not spread from man to man in its acute form it cannot be said to be epidemic in the true sense In its best known form it is contracted through direct contact (usually bite) with infected animals and its clinical nature is partly determined by the portal of entry

In animals in areas where it is endemic it is probably a usual resident of the respiratory or the intestinal tract then when unfavorable environmental factors—poor sanitation extreme weather, shipping—lower the resistance of the hosts it flares up into acute form running either a septicemic often fatal course or assuming a clinical form according to the system of organs affected—pneumonia or enteritis Then the organisms are circulated throughout the flock or the herd in excretions and discharges In its epizootic phases in fowl it may affect one or several species Although the organisms can survive for a time outside the animal this does not adequately account for the sporadicity of the outbreaks Spread by carriers and excreters seems to be more probable Economic losses due to pasteurellosis of fowl, cattle, pigs and buffaloes continue to be great particularly in the tropics

Pasteurellosis in Man

Human infection is rare but is being recognized more frequently (Schipper, 1947 Olsen and Needham 1952 Pizey 1953 Thomas et al, 1953 Bearn et al 1955 Ewan 1955 Byrne et al 1956) *Pasteurella multocida* was identified in 89 of 109 cases reported but since serotyping was not pursued there is doubt about the actual source of the infection in some The organism has been demonstrable in the sputum of 42 patients suffering from bronchiectasis Animal bites were the precursors in 35 cases 28 cat bites 5 dog bites, 1 rabbit bite 1 panther bite Exposure to cattle pigs or rabbit carcasses (muscle in particular) or consumption of infected rabbits has brought on enteritis conjunctivitis (3 cases) blennorrhea appendicular abscesses urinary infection and other manifestations The generalized infection symptoms of which vary widely from protracted recurrent chills and fever to pneumonia and empyema (16 cases) meningitis and puerperal sepsis have been reported mainly from Europe Before development of antimicrobial drugs most of the generalized infections were fatal The mechanism of infections leading to pleurisy empyema and pneumonia remains undetermined Most of the 8 cases of meningitis or brain abscess were sequels to skull fracture sinusitis or mastoiditis the offending organisms having been present in the nasal passages Sinusitis and persistent (up to 6 months) carrying of a pasteurella in the nose of an animal caretaker or meningitis following plastic operation for an abscess on the nose attest to this conclusion Particularly interesting is the fact that *P multocida* apparently may be carried on the mucous membrane of the respiratory tree for months and only after trauma or

Morphology

P pestis appears in the animal body as short round coccoid or large ovoid safety pin shaped bacilli 1 5 to 2 0 μ long and 0 5 to 0 8 μ wide They are gram negative and the typical bipolar staining of single organisms or pairs contrasts with the uniform color of the spherical bodies Chromatic bodies that change during cell division are responsible for the bipolar stain and suggest a nuclear system In organisms grown in cultures bipolar staining is usually not so well seen and on artificial media particularly liquid chainlets and larger rods are common The stain usually recommended for plague work is 1 per cent carbolthionin Wayson s dye mixture (methylene blue with carbolfuchsin) or modifications of Romanovsky s or Giemsa s solutions Peculiar moldlike and yeastlike serpentine forms and feebly staining irregular bladder and ring types are involution forms in environments unsuitable for growth of this bacillus viz spinal fluid of patients with chronic plague meningitis decomposed carcasses of infected animals or 3 per cent sodium chloride agar *P pestis* is surrounded by an envelope particularly when cultured at 37° C It has been thought that the envelope is actually a particularly well developed bacterial capsule But electron microscope studies of colonies of *P pestis* grown on collodion films over agar have shown a layer of structurally undifferentiated material of lower electron density than the bacterial cell This material lying between and among the cells in colonies is easily removed by suspending the bacilli in distilled water or by emulsifying them in saline solution before mounting them This material more abundant in virulent than in avirulent colonies is considered to be a soluble envelope or slime layer to which the nonsomatic antigens are related (Crocker et al 1956)

Plague bacilli do not have flagella and are not motile

Nutrition and Cultivation

The growth of *P pestis* is always slow The lag phase can be reduced by adding the filtrate of a several day culture to the broth Under ideal conditions the average time required for cell division is about 4 hours

The plague bacillus grows densely in well aerated media and under anaerobic conditions On neutral or weakly alkaline hormone casein hydrolysate mineral glucose medium (Sokhev et al 1950 Englesberg and Levy 1954) or blood agar in the presence of a free oxygen supply colonies appear in from 24 to 48 hours As a rule the colonies are slimy and viscous Changes in the cells in the colonies or in the character of growth are not accompanied by changes in virulence or immunogenicity Dissociation is strictly individualistic Smooth and rough colonies can be detected on tryptose agar to which glucose and yeast extract have been added either in obliquely transmitted light or with the aid of triphenyl tetrazolium Grown in broth the smooth colony type produces uniform turbidity the rough brittle type accumulates as a coarse granular sediment with a completely clear supernatant Stalagmites or stalactites form in broth tubes if sterile oil is floated on the surface In culturing *P pestis* on synthetic medium proline phenylalanine and cystine are essential (Rockenmacher et al , 1952)

DL methionine is replaceable by other sulfur compounds (Englesberg 1952) Apparently many strains have dual sulfur requirements they cannot synthesize cysteine and methionine because of breaks in the conversion of sulfate to thiosulfate and cysteine to cystathionine further they lack an alternative pathway for converting the sulfur of methionine to the sulfur of cysteine At 32 C and below an optimal medium must contain phenylalanine valine isoleucine cysteine methionine and hemin For optimal growth at 30 C the basic medium must in addition contain alanine leucine serine threonine biotin and pantothenate Omission of alanine or leucine delays growth and if biotin and pantothenate are omitted a mixture of 20 amino acids is required (Hills and Spurr 1952) Mutation which occurs so readily strain differences and temperatures of cultivation explain the contradictory reports on the nutritional requirements of *P pestis*

Surface cultivation is improved by the addition of 0 1 per cent blood 0 05 per cent sodium sulfite or hematin to the agar Proper media adequately aerated by shaking furnish maximal growth (4 to 5 x 10^9 organisms per ml) on the third day of incubation at 37 C This is followed by massive lysis and release

been held After the large outbreaks in Milan (1630) London (1665) and Marseilles (1721) higher sanitary standards, better housing and legalized prophylactic measures contributed to its retrogression rampant epidemic plague had disappeared from Europe Asia Minor Syria and Palestine by 1843 The modern pandemic originated in Yunnan on the Burma border of China, reached Canton early in 1894 from there was carried by steamer to Hong Kong and thence to Bombay within 2 years In the next 20 years India was the victim of devastating epidemics Since vessels moved in and out of Hong Kong unrestrictedly it became the focus from which plague was disseminated to seaports throughout the world It was in Hong Kong that the causal organism was discovered by a Swiss Alexander Yersin on June 20 1894 (Ogata 1955) On July 7 1894 Kitasato announced finding an organism in plague cases but the one he described was gram positive and slightly motile

After Yersin s discovery of the bacillus India became the most fruitful center of plague work and as the epidemics there increased in severity the British and the German governments sent commissions to Bombay Their investigations published in excellent reports did much to further knowledge of the disease Many observers had noted that rats began to die before human beings were affected and that rodent and human outbreaks were somehow related Ogata in 1897 suggested tentatively that the flea might play a part in transmission and then a little later Paul Louis Simond working in Bombay set down the main facts about the transmission the epidemiology and the control His hypotheses played an important role in the success of the antiplague experiment in San Francisco in 1903 when the U S Public Health Service put them to test The actual part played by the rat was made clear in the reports of the British Plague Research Commission (1906 1917) contact with rats is not followed by plague if fleas are excluded healthy rats can be kept in contact with live infected rats and with rats dead of plague without contracting the infection and the disease is not acquired by ingestion Plague infected rats have been captured in epidemics the world over since the Canton outbreak and have been an important link in the transmission chain in most of them Rat plague was also demonstrated on many ships from 1896 to 1936 That rats are not the only animals to suffer from the disease became apparent when marmotlike animals, the tarabagans (*Arctomys bobac*), were found infected in Transbaikalia and Mongolia in 1895, plague ridden squirrels (*Sciurus palmarum*) appeared in India in 1898 and the South African striped mouse (*Rhabdomys pumilio*) was found infected in 1906 Today the number of rodents known to harbor plague is great and the transition from wild to domestic species has attracted due attention in Russia South Africa, the United States on the Peruvian Ecuadorian frontier and in the Argentine Through recent studies in Iran, Kenya and the Central Provinces of India it appears that plague in order to maintain itself in focal areas requires resistant wild rodents capable of surviving the epizootics

The mechanics of plague transmission from rats to man and among rats themselves was studied by Ogata who injected crushed fleas from an infected rat into mice and thereby produced plague The British Plague Research Commission working in Bombay (1905 1906) showed in experiments that the rat flea *Xenopsylla cheopis* transmits plague from rat to rat How the bacilli multiply in the stomach of the flea and are then regurgitated during the sucking act of the insect was worked out by Bacot and Martin More recently important contributions to the study of fleas involved in sylvatic plague have been made by workers in the U S Public Health Service

Immunization with killed organisms was initiated by Haffkine in 1896 and improved by Sokhey Vaccination with live avirulent cultures was introduced by Otten in Java and has been improved and put into practice in Madagascar South Africa and U S S R Antiplague serum has been used in therapy since the time of Yersin and the potency was improved at the Haffkine Institute The therapeutic value of sulfonamides streptomycin chlortetracycline and chloramphenicol in bubonic and pneumonic plague has been established in field tests in India and Madagascar

Resistance

The plague bacillus is killed by sunlight in 3 or 4 hours it is inactivated in 10 to 15 minutes by heating at 55 C 0 5 per cent phenol or 95 per cent alcohol It remains viable in dried sputum for at least 3 months and in dry flea feces held at room temperature for 5 weeks (E key and Haas, 1940) and its virulence is preserved in rat fleas dried in vacuo *P pestis* is not sensitive to cold cultures and organs held in the icebox retain their virulence for up to 25 years When suspensions of avirulent *P pestis* are lyophilized rapidly and stored at 10 C in 1 per cent sodium glutamate or 5 per cent glucose or lactose or sucrose salt solution from 35 to 65 per cent are viable after 5 years Virulent strains can be preserved by cultivating on 5 per cent rabbit blood agar at 37 C for 3 days the tubes sealed and the material stored at 4 ± ² C

Antigenic Structure

The characterization of the antigens was begun with the identification of a thermolabile envelope and a somatic antigen by Schutze (1932) who used serologic absorption and precipitin tests The envelope antigen develops best at 37 C and withstands brief boiling (Amies 1951) The somatic antigen is heat stable and develops best at 28 to 30 C Quantitative differences in the organisms cultured at 37 C and at room temperature have been revealed by precipitin complement fixation and mouse immunization tests Since 1947 chemical methods have been applied to isolate antigens from cell extracts Baker and his associates (1951) divided the antigens of acetone killed dried virulent plague bacilli into water soluble and water insoluble components Three antigenic entities were defined by their solubility in ammonium sulfate solution (1) a protein carbohydrate complex (Fraction IA) soluble at 0 25 saturation but not at 0 3 (2) a carbohydrate free protein (Fraction IB) soluble at 0 3 and crystallized from 0 3 ammonium saturation (3) a toxic component (Fraction II) Crystalline Fraction IB is an electrophoretically uniform protein By physical chemical and immunologic tests it is identical with Amies antigen Fraction II free from Fraction I is identical with the endotoxin originally described by Rowland The water insoluble fraction or residue is toxic for mice (LD_{50} 0 5 mg per 20 gm mouse) This antigen produces necrosis in animals after subcutaneous inoculation and confers resistance against water extracts or broth filtrates of *P pestis* Extraction of acetone dried cells of *P pestis* with aqueous phenol after removal of proteins by saline has yielded a specific lipopolysaccharide (Davies 1956)

Agar precipitin technics (Oudin Ouchterlony Oakley and Fulthorpe) are now being applied to the analysis of the plague antigens (Landy and Trapani 1954 Chen and Meyer 1955 Ransom et al 1955 Crumpton and Davies 1956) At least 10 lines of precipitation have been recognized and the evidence has been presented that all represent different antigenic molecular species Virulent and avirulent strains contain 7 antigens in the Oudin test 4 are thermolabile and 2 thermostable the remaining antigen is haptenized Fraction I By using the diffusion precipitin technic in conjunction with animal experiments and immunologic tests the lines of precipitation corresponding to antigens having some known physiologic property have been established (1) the envelope antigen which protects animals and man from fatal plague (2) the plague toxin (3) a polysaccharide which plays no part in the protection of mice or guinea pigs According to antigen studies by Burrows and Bacon (1956) *P pestis* can develop resistance to phagocytosis in the absence of visible envelope or capsule Fully virulent strains have the ability to produce two antigens—Fraction I and V or W demonstrable by the agar diffusion precipitation technic Antiserum containing demonstrable amounts of V and W antibodies can render resistant organisms sensitive to phagocytosis All virulent strains studied have elaborated these antigens whereas with one exception (strain EV 76) avirulent strains have not Some avirulent strains lack one or more of the antigens One such strain (TRU) neither protective for mice nor toxic produces only 5 of the 10 precipitation lines Maximum production of the 3 important antigens occurs when the organisms are grown at 37° C, although the optimal growth temperature is 28 C Most avirulent

into the medium of at least 3 components of *P pestis* envelope (Fraction I), somatic antigens and toxin (Englesberg and Levy, 1954)

Isolated cells of *P pestis* will not grow under aerobic conditions unless hemin or some other substance that destroys hydrogen per oxide is added to the medium (Herbert 1949) When cultivated on desoxycholate agar for 48 hours at 37° C plague bacilli grow scantily in reddish pinpoint colonies

Biochemical Activities

Pyruvic acid accumulates in well aerated cultures of *P pestis* in glucose or mannitol mineral base medium supplemented by phenyl alanine and cystine Under anaerobic condi tions in complex medium containing glucose the chief products of fermentation are lactic and acetic acids and ethyl alcohol carbon dioxide formic acid succinic acid and a small amount of pyruvic acid are also pro duced but no hydrogen glycerol or 2 3 butylene glycol and only traces of acetyl methyl carbinol or diacetyl L-serine is oxi dized rapidly by resting cells through pyruvate and acetate to carbon dioxide Glucose galac tose fructose mannitol and mannose and as a rule arabinose maltose and xylose are fer mented while lactose sucrose raffinose dulci tol inulin and as a rule rhamnose are not The over all fermentation pattern of glucose reveals that *P pestis* contains enzymes of both the Embden Meyerhof and the hexose mono phosphate shunt pathway the latter operates during growth The CO produced during fer mentation arises from the dismutation of pyruvate (Santer and Ajl 1954 1955)

All *P pestis* strains glycerol positive or glycerol negative give off rhamnose ferment ing mutants Rhamnose utilization has quali tative effects on motility urease production glycerol fermentation and growth on desoxy cholate agar Mutation to R+ does not con vert it into *P pseudotuberculosis*

P pestis was long considered a homogeneous species but recently 3 biologic varieties of some epidemiologic significance have been dis tinguished Berlin and Borzenkov first sepa rated 2 types one continental glycerol posi tive the other, oceanic, glycerol negative The continental type has since been divided into 2 subtypes according to whether or not it re duces nitrates to nitrites Devignat (1951) has proposed the following classification

Pasteurella pestis

var *orientalis*	glycerol −	nitrite +
var *antiqua*	glycerol +	nitrite +
var *mediaevalis*	glycerol +	nitrite −

The stability of *P pestis* with respect to glycerol is incontestable, but the constancy of results of tests for reduction of nitrates to nitrites cannot be accepted without reserva tion No fundamental differences between glycerol positive and glycerol negative strains have been found, and the test is primarily valuable for the solution of nosogeographic problems The Oriental variety (oceanic type) is found in India Burma, South China Europe, the United States South America Ceylon Egypt Java, North Africa Senegal Sumatra, Thailand and Madagascar It pro voked the modern pandemic which originated in Yunnan in 1894 spread overseas into the 5 continents the rat and rat fleas having been the original and the subsequent agents of transmission The antiqua variety (conti nental type) seems to have been the cause in the ancient centers of pestilence in Southeast Russia Central Asia Mongolia Manchuria Transbaikalia and Central Africa where the organism is perpetuated through wild rodents The medieval variety is found south of the Caspian Sea lower Volga region in the Iranian Kurdistan Turkey and Iraq

Catalase activity has been recommended for determining the virulence of plague strains in vitro Ribonucleic acid is decomposed en zymatically by living *P pestis* and by prepa rations free from bacterial cells *P pestis* in contrast with *P pseudotuberculosis* does not produce acid in Ferguson s synthetic medium or Difco slants with ordinary urea Urea is hydrolyzed rapidly by *P pseudotuberculosis* Lysis by bacteriophages isolated from rat feces rat lymph nodes sewage and various other sources is a property common to all strains and varieties of *P pestis* This homo- geneity in susceptibility to lysis by phage is unique Hemolysis does not occur on blood plates It is generally agreed that *P pestis* produces neither H S nor indole and does not bring about changes in milk Reduction of nitrates is variable that of methylene blue and Janus green is distinct Broth cultures become alkaline and may reach pH 8 at the end of 9 weeks

from virulent or avirulent pasteurella strains

Hyperimmunization of rabbits and monkeys with living virulent or avirulent plague bacilli has given rise to antibodies that neutralize 10 LD$_{50}$ of toxin in 0 01 to 0 0025 ml of serum when tested by the intraperitoneal route The neutralizing antibodies react quantitatively with the pure toxin in flocculation systems but the antibody toxin complex is not stable On intravenous administration dissociation occurs Antitoxic serum injected daily has not influenced the course of the infection in mice Serum devoid of envelope antibodies has not limited its progress or protected the vascular system against the toxin slowly released in the course of infection Purified toxin sensitizes tannic acid treated erythrocytes and this provides a useful procedure for determining serum antitoxin levels Using specific antitoxin in complement fixation tests it is possible to measure the concentration of toxin in cultures or toxoid Prolonged immunization of mice with partially purified toxin confers some protection against *P pestis* infection complement fixing and hemagglutinating antibodies against envelope antigen in high titers are demonstrable in the peripheral blood serum Immunogenicity of a single strain apparently is not related to its toxicity (Chen and Meyer 1955)

Distribution and Pathogenicity

In bubonic plague in man *P pestis* may be demonstrated in primary vesicles and with certainty in the gelatinous edema fluid surrounding or within lymph nodes Bacteremia is the rule even in mild bubonic infection it may disappear as early as the second day or it may persist for 10 days in severe infections At autopsy *P pestis* is regularly found in heart blood lymph nodes or spleen but especially in bone marrow and secondary pneumonic lesions In primary pneumonic plague the bacilli are present in sputum before they can be detected in the blood

Now 372 animal species have been listed as known or suspected hosts (Pollitzer 1954 Macchiavello 1954) Among the domestic animals cats are moderately susceptible dogs usually are refractory chickens are not absolutely resistant to experimental infection It has been claimed that sheep and camels can be infected

The rodents usually used in experimental work—guinea pigs white mice multimammate mice cotton rats rabbits and rats—are all susceptible to plague Experimental plague can be produced easily in guinea pigs by any route Most of these animals can be infected with only a few organisms but there may be seasonal and genetic variations Guinea pigs are desirable subjects for diagnostic work because the lesions that develop are so characteristic Rabbits and monkeys are variably susceptible Wild rodents are less reliable because their background with respect to plague is unknown White rats are not as susceptible as other *Muridae* and lesions produced in them are not as characteristic as those in the guinea pig

During the past 5 years the work of Burrows and Bacon (1955 1956) has indicated that for full virulence a strain of *P pestis* must have all of the following capabilities (1) to elaborate envelope antigen (2) to be highly toxigenic (3) to synthesize purines (4) to produce pigmented colonies on a defined medium containing hemin (5) to develop resistance to phagocytosis (mouse polymorphonuclear cells) in the absence of visible capsulation (6) to synthesize antigens V and W The mechanism by which virulent cells resist phagocytosis remains to be learned In particular the role of the antiphagocytic antigens V and W in mouse grown M phase bacilli in contrast with those grown in vitro requires elucidation in the mouse and other animals In chick embryos highly virulent plague strains proliferate freely avirulent strains sparingly The former in small numbers kill the embryo while the latter administered in sublethal doses persist in embryonic organs until hatching time and sometimes for 3 to 4 days after the chicks hatch (Buddingh and Womack 1941 Jawetz and Meyer 1944b)

The virulence of most *P pestis* strains freshly isolated from human or rodent infections is of a high order Barber found that 6 of 9 guinea pigs and 2 of 12 monkeys receiving 1 virulent bacillus died of plague Methods of measuring virulence in mice (Sokhey 1939) and guinea pigs (Otten 1941) have been standardized Organisms from cultures and occasionally from chronic lesions in rodents may show a complete absence of virulence Some strains losing part of their virulence will regain it on passage

strains produce only traces of the protective antigen at 37° C Cells of virulent strains grown at 20° C contain only traces of these 3 antigens

Of the 10 or more different antigenic molecular species the envelope antigen is the protective antigen for all experimental animals and in all probability for man

Antiserum prepared with the protective envelope antigen agglutinate all plague bacilli with envelopes in woolly flaky aggregates so called envelope type of agglutination Strains with little or no envelope are agglutinated by antiserum prepared against whole plague bacilli the aggregates are fine hard granules of the so called somatic type Such serum agglutinates *P pseudotuberculosis* because it contains antibodies against the common somatic antigens but serum against *P pseudotuberculosis* will not agglutinate plague bacilli grown at 37° C because the somatic antigens are protected by the envelope antigen (Bhatnagar 1940) Cross reactions are obtained in precipitin tests with aqueous ether extracts of plague bacilli and antipseudotuberculosis serum (Larson et al , 1951)

A specific high titered anti envelope serum detects quantitatively the envelope antigen in vaccines and in tissue extracts of animals dead from plague in the complement fixation test Antibodies to the envelope antigen are detectable by the same procedure in convalescent human serum and that of animals immunized with living avirulent or killed organisms or pure envelope antigen (Chen and Meyer 1952)

Red cells treated with filtered broth cultures containing a polysaccharide antigen are specifically agglutinated by antiserum of animals or man injected with whole cell antigens but not with envelope antigen Purified envelope antigen is readily adsorbed to red cells after preliminary treatment with tannic acid Erythrocytes so treated are specifically agglutinated by antiplague serum The hemagglutination test is from 20 to 50 times as sensitive as the complement fixation test and is as specific

Circulating complement fixing hemagglutinating, agglutinating and mouse protective antibodies in the serum of immunized guinea pigs are reliable indications of their immunity to infection

Toxin

Fresh suspensions of live virulent plague bacilli (500 000,000 to 1,000 000 000 cells in 0 5 ml) injected intravenously into mice are fatal within 4 to 12 hours Suspensions of dead bacilli or cell free aqueous extracts of such suspensions are also fatal to mice This endotoxinlike poison (Girard, 1955), injurious to mice (LD_{50} 0 017 mg /Kg) and rats in small doses, to rabbits (LD_{50} 40 mg /Kg) and monkeys in larger doses but to guinea pigs only in very large doses (LD_{50} 80 to 100 mg /Kg) is probably identical with the broth filtrate toxin first described by Markl It can be partly purified by ammonium sulfate precipitation (Baker et al 1952) and purified by combined salt and alcohol precipitation or by continuous flow paper ionophoresis (Ajl et al 1955, Spivack and Karler, 1958) The crude toxin is obtained by a 3 step precipitation with ammonium sulfate from avirulent or virulent strains The resulting material is relatively free from envelope antigen, but the Oudin tests show the presence of 1 to 3 contaminating antigens Temperatures of 56 C for 4 hours partially destroy it and at 75° C for 5 minutes the destruction is complete it is stable over ranges of pH 5 to 8 and remains so for 4 months at 4 C It becomes atoxic at pH 3 or less and at pH 10 Formalin (0 1%) converts it into a toxoid that retains its antigenicity, while phenol in excess of 0 5 per cent alcohol mercuric borate and prolonged treatment with chloroform destroy it Tetracyclines protect mice against 2 to 4 LD_{50} if injected in advance of the toxin

The toxin acts mainly on the peripheral vascular system causing hemoconcentration shock and glycogenolysis in the liver as well as parenchymal injury at first in the liver and then in the kidneys On intradermal administration edema is often followed by tissue necrosis The toxin exerts no selective action on the central nervous system or the heart The Shwartzman phenomenon cannot be elicited with the toxin nor with any of the other antigens Extracts from virulent or avirulent strains contain a factor that enhances spreading and capillary permeability It is highly antigenic Inoculation of rabbits with purified toxin in adjuvant incites production of antitoxin capable of neutralizing toxin extracted

and bone marrow causing septicemic plague In experimental plague a highly susceptible animal that receives direct intravascular injection of bacilli through the bite of a flea may contract immediate septicemic plague with slight or only secondary involvement of the lymph nodes Primary foci of bacillary multiplication in the spleen and bone marrow constantly flood the blood stream with organisms and toxins in the septic fulminating variety In the course of bacteremia bacilli may localize secondarily in the skin and form pustules (carbuncular plague) Wherever plague bacilli multiply in enormous numbers coagulation necrosis with hemorrhagic inflammations seriously damages the lymph nodes and focal areas in the spleen and the liver These processes undergo slow resolution or suppuration and may contain viable bacilli for many weeks Lymph nodes may heal by softening and ulceration in some instances there is gangrene (Jawetz and Meyer 1944a)

Primary pneumonic plague is due to infection through some portion of the bronchi or the bronchioles contiguous to lung tissue At least experimental intranasal infections in mice and guinea pigs give rise to localized peribronchial and perivascular inflammation in tissues adjacent to bronchioles and then to more diffuse inflammation throughout the lungs

The LD_{50} is much greater when tested by the respiratory route than by the subcutaneous Two types of plague can develop in the respiratory tract of mice or guinea pigs depending on the size of the particle introduced Small particles initiate bronchopneumonia which leads to septicemia and death with pulmonary edema Large particles establish septicemia and death results more quickly without pneumonia (Druett et al 1956) In a cross infection experiment in which normal monkeys were exposed to monkeys with primary pneumonia in a divided cage in which only the top part was open the exposed monkeys contracted septicemic not pneumonic plague The portal was the lymphatic tissues of the oropharynx In man this type of infection has been described as tonsillar plague (Meyer 1957) Primary fatal pneumonic infection in monkeys can be brought about by intratracheal instillation of 100 virulent *P pestis* inhalation of about 20 000 cells has induced a chronic nonfatal pneumonic process in which virulent bacilli have remained viable for 40 days The pathogenesis of secondary lung involvement after bubonic infection in man and not infrequently in partially resistant guinea pigs surviving cutaneous or subcutaneous infection is not clearly understood It may be that emboli from focal lesions in lymph nodes the liver or the spleen or agglutinated plague bacilli carried by the blood stream lodge in the pulmonary capillaries weakened by the toxin (Meyer 1957)

The characteristic picture at autopsy is engorgement hemorrhage and enlargement of the lymph nodes and extravasation into the perinodular tissues In some severe infections the nodes may be affected either by serous or serosanguineous effusion when they are soft and colored purple or plum purple or by complete hemorrhagic infarction In others there may be exudative edema of adipose tissue fascia muscles and nerve sheaths and the hard nodes may be studded with yellowish granules or colored with bright red extravasated blood When the disease is protracted necrotic foci and yellowish red pus are found in the parenchyma of the nodes secondary buboes are often affected similarly The spleen is enlarged and congested on section the deep red or purple pulp looks granular The liver may be enlarged or engorged its parenchyma is always soft and affected by cloudy swelling or fatty degeneration Straw colored fluid fills the pericardial sac There are ecchymoses on the pericardium and the endocardium the myocardium is pale soft friable and degenerated The blood is coagulated into soft clots or is semifluid Characteristic of the disease is the distention of veins and small blood vessels accompanied by large and small hemorrhages

In all types of human plague the lungs are congested and edematous the lesions may be mistaken for pneumonia in secondary plague pneumonia the areas of consolidation are reddish gray and surrounded by a distinct ring of engorgement Consolidated areas in animals are usually yellow Regional lymph nodes are enlarged and soft Punctate hemorrhages occur in the stomach and the intestines The swollen kidneys are purplish soft and sometimes studded with necrotic foci Although frequently normal in man the adrenal glands of rodents are always enlarged showing cortical hemorrhages when the animal has succumbed to acute plague

through susceptible animals Many attempts have been made to reduce virulence by growing the bacilli under unfavorable conditions (e g, in alcohol broth) selecting at random colonies from virulent cultures refrigerated or incubated at 32 C for some time or by passage through murine animals Although some lose virulence spontaneously natural dissociants more often retain the characteristics including the *immunogenicity of parent* strains while forced dissociation mutilates the descendant by impairing its immunogenicity and destroying the envelope Avirulent strains with qualities that recommend them for use in vaccines have been isolated from cultures held in deep agar slants for 5 to 6 years Many strains cultivated on a chemically defined medium containing hemin form dark brown colonies by absorbing the hemin On prolonged incubation pigmented colonies produce nonpigmented secondary colonies of reduced virulence for mice Nonpigmented mutants of avirulent pigmented strains remain avirulent even in the presence of iron compounds Fully virulent strains can derive adequate supplies of iron to permit the development of large populations in vivo (Jackson and Burrows 1956)

PATHOGENESIS

Essential for development of bubonic plague in man is the primary rat flea rat transmission cycle The flea becomes infected from the blood of a sick rat which in the terminal bacteremia may contain 10^7 bacilli per cu mm of blood (Douglas and Wheeler 1943) These bacilli multiply in the midgut of the flea and massive infection develops in the proventriculus blocking the pharynx and the esophagus When the flea attempts to take its next blood meal from 25 000 to 100 000 bacilli are regurgitated via the insect s proboscis into the skin or the capillaries of the new mammalian host Other rodents may replace the rat in this cycle The infection of man is an offshoot rarely are there enough bacilli in human blood to infect fleas Fleas ordinarily infesting man primarily (especially *Pulex irritans*) are usually not good transmitters but under certain circumstances human fleas can transmit bubonic plague from man to man directly or indirectly when the infestation is extremely heavy favored by thick layers of clothing and rare ablutions as it has been in Morocco

When plague bacilli become localized in the lungs and produce pneumonia droplets from the respiratory tract are highly infectious Then primary pneumonic plague may spread readily from man to man, and a true epidemic occurs There are several other less probable means of infection—inhalation of dust of infected flea feces inducing primary plague pneumonia the eating of undercooked plague infected marmots (in Manchuria), the killing of fleas by biting them or biting off the heads of infected rodents

P pestis may enter the body via the blood the skin the conjunctiva or mucous membranes of the respiratory or the digestive tracts In the infected rodent or man both the lymph and the blood stream may act as pathways for the bacillus

The plague bacillus injected into the skin of the mammalian host may be held up at the site the local vesicle or pustule represents the first line of defense This symptomless form reflects considerable immunity Recent findings in Madagascar (Payne et al 1956) suggest that this form may be more common than it had been thought to be Inflammation of the lymph channels leading from the local lesion to the lymph nodes is caused by toxins released by destroyed organisms not by the multiplying bacilli If the *P pestis* passes the skin barrier it is held up at the first group of lymph nodes to which the lymphatic vessels pass the nodes enlarge and are embedded in a gelatinous periglandular inflammatory edema The infection may be arrested at this stage with only mild constitutional symptoms (pestis minor) If the bacilli pass this second line of defense they reach the *secondary* lymph nodes draining the area of inflammation and small numbers pass into the blood stream and from there to the spleen the live and other lymph nodes An interplay between antibodies and fixed tissue leukocytes and possibly other factors may limit this bacteremia to showers of organisms Nevertheless generalized infection in many parts of the body creates grave constitutional symptoms If the immunity is inherently inadequate or has been damaged by toxins plague bacilli not only multiply intravascularly but are constantly washed into the circulation from the spleen

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Judged by the pathologic changes, bubonic plague is a disease of the lymphatic and the vascular systems in which numerous bacilli and their toxins cause inflammation, coagulation and necrosis Primary pneumonic plague differs in that hemorrhagic extravasation is concentrated in the lungs

IMMUNITY

The immunity that follows infection is relative Patrick Russel recorded 28 reinfections in 4 400 cases

Resistance of wild rats or squirrels to experimental plague depends on the extent of endemic disease in the localities they had inhabited (Meyer 1942) In India where plague had been prevalent only 7 9 per cent of the rats tested were susceptible to experimental plague (Sokhey and Chitre 1937) But in Madras City where the rodent population had not been exposed 91 7 per cent were susceptible

Prevention of plague by protective inoculation has been studied ever since Haffkine in 1896 made extensive animal and human experiments with heat killed broth antigens The attack rate was not impressively reduced after administration of one dose of Haffkine antigen But the mortality rate of immunized sulfa treated patients was less than half that of the corresponding unimmunized group and among those not specifically treated, fewer of the immunized died (Patel and Rebello 1948) Killed plague bacilli protect mice guinea pigs monkeys and probably man against infection The protective antigen is not destroyed by alcohol, acetone formalin, phenol or heat and the efficacy is greatly enhanced by adjuvant

French and Dutch workers tested the killed preparations on guinea pigs and wild rats—the test animals most susceptible to plague—and the results led them to the conclusion that this type of immunization would not protect man Therefore they spent their efforts on the development of avirulent organism vaccines In field tests in Java and Madagascar vaccines composed of living avirulent bacilli have shown promise In endemic areas where native populations are heavily exposed at times a preparation that can be given in a single dose has administrative and economic advantages The annual reports of the Pasteur Institute at Tananarive, Madagascar, have shown a striking decline in the number of cases—from 3 035 in 1935 to 14 in 1954 55—under the impact of from 600 000 to 800 000 annual vaccinations with living avirulent plague vaccine

Immunization experiments over the years have consistently suggested the existence of a specific antigen that protects the guinea pig against plague This apparent host specificity has now been explained The protein envelope antigen confers protection to the guinea pig when incorporated in oil adjuvant in doses of 1 to 50 micrograms, in doses of 1 mg or more in oil adjuvant it produces immunoparalysis No evidence of a qualitatively distinct guinea pig protective antigen could be found All the early tests with killed organism preparations were inoculated as aqueous suspensions or lysates and in doses that exceeded the optimum of the immunogenic antigen (Spivack et al, 1958)

In the United States attention has been focused on the development of killed immunogens because vaccines made with living avirulent *P pestis* have the following disadvantages With the usual dose (1×10^9 organisms) the systemic reactions are severe The antibody response is moderate even to a booster dose The vaccine kept in a fluid state at 5° C loses its antigenicity within 1 to 2 weeks and when lyophilized the protectiveness is decreased Formalin killed bacterial suspensions produce milder local and systemic reactions

Since the protective antigen in the plague bacillus is the envelope antigen efforts have also been made to develop this for immunization but it has not been evaluated under field conditions This antigen freed from toxin and given in adjuvant has prompted the production of mouse protective antibodies in higher levels than living vaccines have

Any known type of immunization has th following limitations The immunity is short A single subcutaneous vaccination does not protect against direct aerogenic infection Some persons who have been vaccinated repeatedly have died of plague Whether the envelope antigen in adjuvant or other killed vaccines are as protective as live attenuated vaccines cannot be answered directly Natural exposure to epidemic chances is becoming in

creasingly difficult because the disease is receding to population groups where reporting and diagnosis often leave accuracy and completeness to be desired Measurement of the antibody reactions indicates the effectiveness of immunization and this is being used as tests and opportunities become available

The sera plasma and other body fluids of immune animals contain agglutinins precipitins complement fixing hemagglutinating (against the envelope and toxin) and mouse protective antibodies but they can neither lyze nor destroy *P pestis* in vitro or in vivo in the absence of phagocytic cells Whole blood of plague immune animals destroys many more plague bacilli than that of normal animals does Only admixtures of immune serum and immune cells destroy a significant number of organisms Immune animals fix the bulk of bacilli at the site of injection however the protective mechanism does not depend on lymphatic blockage and deposition of fibrin network Cytograms of the local inflammatory exudates examined with a phase contrast microscope have shown that in the animal with an acquired immunity the capsule of the plague bacillus becomes morphologically altered through the formation of a fine precipitate Thereafter the bacilli are readily phagocytized and are lysed within polymorphonuclear leukocytes The immunity mechanism in man and animals apparently consists of a humoral phase responsible for removing the antiphagocytic property of the capsule followed by ingestion and intracellular disposal of the phagocytized bacilli Excessive soluble antigen may block the action of the antibodies and endotoxins liberated by the lysed organisms may poison and paralyze the entire immunity mechanism (Meyer 1950)

Significant and confirmatory of the observations on animals are the observations that a single inoculation of living attenuated *P pestis* strain E V elicits demonstrable envelope antibody and antitoxin in relatively few persons Annual injection of the vaccine progressively enhances the production and the maintenance of the antibodies by the individual and by the group (Girard 1955) As little as 11 micrograms of intradermally injected envelope antigen elicits an appreciable antibody response in most previously vaccinated but not in unvaccinated persons (Payne et al 1956) Mouse protective antibodies of high titer appear by the 30th day in the sera of patients that have recovered from pneumonic plague (McCrumb et al 1953)

Yersin and his colleagues were the first to demonstrate that sera from rabbits immunized with inactivated cultures effectively protect other animals against infection These results established the principle of passive immunization and gave hope for serum prophylaxis and serum therapy The effectiveness of serum has been proved repeatedly mortality rates among the treated in one series was approximately 28 per cent and among the untreated 58 per cent To be effective serum must be given intravenously early Attempts to develop potent serum which causes no alarming reactions have met with varying success A rabbit antiplague globulin solution prepared in the United States is pyrogen free and potent

Diagnosis

The diagnosis of sporadic cases is likely to be missed Early diagnosis is of the greatest importance to the patient and the attending staff as well as to his family and community In endemic regions plague must be remembered as a diagnostic possibility A typical case of severe bubonic or septicemic plague presents a characteristic picture sudden onset high temperature rapid pulse white coating of the tongue nervous symptoms varying from restlessness to great prostration and fatigue bloated appearance and conjunctival suffusion slurred speech and staggering gait apathy and mental confusion Eventually there is pain in the groin the armpit or the neck where the bubo appears Intense pain directs the attention of the patient to the inflamed node which may remain small hard and tense but more frequently enlarges to the size of a walnut or a goose egg and is embedded in boggy edema In the septic variety nervous and cerebral symptoms supervene with striking rapidity although the temperature is rarely above 100° F epistaxis hematuria and involuntary evacuation appear in rapid succession Pneumonic plague begins with rigor malaise severe headache nausea vomiting and general pain temperature from 102 to 105 F difficult and hurried breathing cough and expectoration The sputum watery and frothy becomes

blood tinged but is rarely viscid or rusty, as it would be in acute pneumonia Diagnosis requires laboratory assistance

Laboratory Diagnosis Recently, this subject has been well covered in complete detail by an international group of plague workers (Baltazard et al 1956)

The bubo should be punctured in its early stages with an 18 gauge needle mounted on a well fitted 5 to 10 ml syringe and a little gelatinous edema fluid aspirated The person taking the specimen should wear rubber gloves and mask The skin over the bubo is painted with iodine and the puncture wound is disinfected with absolute alcohol Care must be taken in expelling the few drops of fluid on blood plates or blood agar slants and in making thin films on several slides since any spray could carry bacilli into the atmosphere Polychromatic stain reveals characteristic *P pestis* Small delicate colonies develop in medium incubated at 30° C for 24 to 48 hours Inexperienced persons should not diagnose plague on the basis of microscopic examinations alone contaminants such as *Escherichia* or *Salmonella* also give bipolar reactions The culture may be identified quickly by means of specific bacteriophage or preferably by an agglutination test with potent antiserum For agglutination tests the bacterial growth is emulsified in a 0 45 per cent saline solution containing 1 per cent formalin The bacilli are killed in from 30 minutes to 2 hours at 37° C The even suspensions free from precipitates are added to the serum Flocculent agglutination usually appears within 2 hours at 37° C Final identification is made by biochemical tests and guinea pig inoculations Infection may be accomplished by rubbing some of the culture intracutaneously or by subcutaneously injecting a few drops of a heavy suspension The animals usually die within 3 to 8 days with characteristic local and general lesions

Blood cultures should be prepared by collecting at least 5 ml of blood from the cubital vein and combining it with 2 ml of sterile 5 per cent solution of sodium citrate Enrichments are made in cystine broth or 0 25 ml of the citrated blood is distributed on several blood agar slants or plates to reveal any septicemia and its degree More than 10 colonies indicate severe septicemia fewer, temporary showers of mild septicemia In the enrichment broth culture chainlike aggregates of ovoid bacilli may confuse the microscopic picture An agglutination test with the formalin treated broth culture is useful

Sputum should be examined both microscopically and culturally on blood or gentian violet (1 70 000) plates There is usually no difficulty in recognizing the plague bacilli which are present in great numbers

Every state in the U S authorizes physicians or health officers to demand an autopsy if plague is suspected Plague infected material must be shipped in properly prepared containers with double screw tops The material should include heart blood portions of bubo, spleen and bone marrow In areas where autopsy can be refused plague envelope antigen can be detected in a modified precipitin test (Hoyer and Courdurier 1954) or by animal inoculation of suspensions of tissue plugs obtained by thrusting a hollow needle into the lung the liver and buboes (Girard 1952) The precipitin test or a further modification with fluorescent antibody may be used to examine decayed or mummified carcasses of rodents

TREATMENT

Treatment has progressed from almost hopeless to highly successful within the last decade Chemotherapy has displaced serum and bacteriophage therapy The latter once used extensively in the French colonie yielded little clinical benefit Sulfonamides have been given experimental and clinical trial in bubonic and septicemic plague The reduction in mortality rate the moderate cost and the oral route of administration highly recommend these drugs Extensive field trials in India give sulfadiazine preference over other sulfa derivatives tested including sulfamerazine The exact value of sulfonamides in preventing contagious primary pneumonic plague is not known Since the sulfonamides inhibit bacterial multiplication and specific plague antiserum enhances the immunity—the two essentials for eliminating infection—it is not surprising that this combination reduces the mortality rate of sulfonamide treated plague still further

Streptomycin is the most effective therapeutic agent thus far tested in the treatment of experimental bubonic septicemic and pneumonic plague in mice and guinea pigs When given in adequately large doses chlortetracycline and chloramphenicol are also effective Penicillin is useless However whereas strepto-

mycin effects cures within 48 hours chlortetracycline and chloramphenicol therapy is complicated by relapse if such treatment is not continued for 7 to 10 days The mode of action appears to differ from that of streptomycin In India streptomycin has dramatically reduced mortality rates from 50 to 10 per cent in both bubonic and septicemic plague The most spectacular effect is in pneumonic plague formerly irremediable (McCrumb et al 1953) About 10 hours after the beginning of treatment virulent *P pestis* have disappeared from the sputum and the risk of contamination of the attendant staff and those associated with the sick person is reduced rapidly Very rarely a strain has developed resistance to streptomycin within 48 hours after treatment of bubonic and pneumonic plague was begun chloramphenicol may serve as an excellent alternate Care must be taken in the early treatment with massive doses of streptomycin in combination with other antimicrobial drugs This may eliminate *P pestis* from the tissues too rapidly releasing large quantities of toxin causing sudden death If it were available a specific antitoxic serum containing no envelope antibodies would help to counteract this hypertoxic form of plague

Epidemiology

Plague is endemic in certain parts of India and is constantly present in Kurdistan Burma Java China Madagascar and South Central and East Africa It occurs sporadically in Egypt North Africa (Tunis) Iraq Iran Siam and Indo-China (Pollitzer 1954) Local rat epizootics occasionally accompanied by a few human cases have been noted in European seaports from time to time the most recent ones being on Malta and Sardinia The plague foci in America constitute a potential source of epidemic plague Watchful unceasing research and inspection are always needed in the enzootic plague areas if large outbreaks are to be avoided The United States has known rat and human plague since the beginning of the century San Francisco (1900 and 1907) the Gulf States—Louisiana Texas and Florida (1914 to 1920)—and Los Angeles (*1924 to 1952*) were affected in turn After a 20 year hiatus the rat disease erupted in Tacoma Wash in 1943 and 1944 Rural endemic foci are established on Maui and Hawaii Human plague cases have been few since 1934 in California and the 15 Western States extending as far east as Kansas Oklahoma and Texas where in 138 counties pockets of enzootic and epizootic plague have been recognized in ground squirrels chipmunks prairie dogs wood rats and harvest mice Since 1908 70 cases (45 deaths) have been traced to wild rodents in these areas (Link 1955)

In the epidemiology of plague two fundamentally different forms should be considered (1) bubonic, or zootic plague produced usually by the bite of plague infected insect vectors mainly rodent fleas and (2) primary pneumonic or demic plague due to spread from man to man The basic prerequisite for the single cases outbreaks or epidemics is its maintenance in the rodent population Periodic epizootics exterminating a variable proportion of the rodents offer opportunity for man to become infected either directly or more often by the intermediation of fleas

There are two main reservoirs urban plague maintained by commensal rats and mice and rural plague maintained by wild rodents Either may give rise to bubonic or pneumonic plague the epidemiology under going corresponding changes Urban plague occurs in densely populated unsanitary areas spreads along overland routes and crosses oceans in ship cargoes Primarily bubonic it tenaciously fixes itself to human habitations and spreads epidemically Under certain environmental conditions more frequently in cold countries it becomes pneumonic developing into epidemics through man to-man transmission as it did in Los Angeles in 1924 Rural plague among hunters workers in woods and children transmitted through rodent bites or handling and perhaps infrequently by insect bites is sporadic bubonic plague which may give rise to small or large epidemics of pneumonic plague Classic examples are the Manchurian epidemics of 1910 1911 and 1920-1921 which claimed 60 000 and 8 502 victims respectively (Pollitzer 1954)

The seasonal spread is influenced by temperature and humidity a moderate temperature 60 F and a moderately high relative humidity indicated by a saturation *deficiency*

of less than 10 millibars are the most favorable for checking it The disease tends to occur during summer months in cooler climates and in the spring months in the hot dry climates of the subtropics in tropical countries where the temperature is fairly constant throughout the year, the incidence follows the humidity curve Many complex factors control the periodic waves, but fluctuations in endemic areas are always associated with variations in the climate As the rodent population in urban areas is reduced by a rampant epizootic and the fleas seek new hosts persons of all ages and both sexes contract bubonic plague General health nutritional status and living conditions, including overcrowding are determining factors in the mortality rates there does not seem to be any racial immunity Doctors and nurses who care for patients with pneumonic plague and other contacts including funeral attendants are liable to attack by the pneumonic form In the rural plague regions men and children who handle or play with wild rodents are attacked more frequently

Strains of *P pestis* secured from man rats wild rodents and fleas from every corner of the earth are biologically identical and remarkably homogeneous in virulence and infectiousness Variations in the severity of pneumonic epidemics are not attributable to a specific pneumotropism

The rodents fall into 2 main groups (1) *Muridae* the rats and the mice living close to man and (2) wild rodents—*Caviidae Dipodidae Cricetinae Gerbillinae Microtinae Murinae Sciuridae* and *Leporidae* (Macchiavello 1954)

Epizootics among the large gray rats (*Rattus norvegicus*) the black domestic rats (*Rattus rattus alexandrinus*) and occasionally others such as house rats (*Rattus griseiventer*) and field rats (*Rattus diardii* in Java and *Rattus hawaiensis* in Hawaii) are influenced by the natural and acquired susceptibility of the individual rat the density of the rat population and the habits which by inclination or opportunity bring the species into close association with man

Plague may be introduced into a rat population by spread from one section of a city to another, by spread at a distance through transportation of rats and rat fleas along lines of communication with merchandise or by partial migration of rodents as in India (Sharif, 1951) Unless there is a considerable population of infected *Rattus rattus* plague is never transmitted to man to any serious extent In San Francisco in 1907 27 (2 69%) of 1,002 live rats examined were infected 56 human cases of plague with 25 deaths were reported during that time By January, after a rigorous control campaign despite an incidence of 1 11 per cent among another tested group of rats of the city, only 2 cases of human plague were listed Rats surviving the epizootics have acquired resistance to infection High immunity rates for example over 50 per cent in San Francisco and over 90 per cent in Bombay, exert a fundamental influence and in some areas may even lead to the eventual disappearance of plague Epizootics cannot arise until a new generation of susceptible rats has grown up It need hardly be emphasized that where houses are mainly constructed of soft material and where garbage is disposed of carelessly conditions are ideal for rat multiplication and potentially for explosive outbreaks of plague

Endemic plague foci maintained by burrowing hibernating rodents such as tarabagans *Citellus* varieties spermophils and mice are found in the brush the deserts and the mountains of Manchuria, the Buriat Mongol Republic Transcaucasia and Southeastern Russia foci exist in South Africa among gerbils and multimammate mice (Davis 1953) in Argentina among *Microcavia graomys* and in squirrels along the Peruvian Ecuadorian frontier In the United States rural plague was discovered in 1908 and since 1934 epizootics have broken out among squirrels prairie dogs rabbits and pack rats in 15 Western States The collection of ectoparasites from sick and healthy rodents and injection of emulsions of these ectoparasites into healthy guinea pig has revealed enzootic and epizootic plague among 50 rodent species

Recurrence of plague in commensal rats in countries where the principal natural reservoirs are squirrels or gerbils without notable repercussions in the nearby human populations is not uncommon Plague in order to maintain itself in focal rural areas requires resistant wild rodents to survive epizootics It is probable that they perpetuate the infection and their fleas may spread it to commensal rodents Plague cannot perpetuate itself without susceptible species capable of rekindling it Complex ecologic factors in the habitual niches filled with hosts parasites and

vectors in the wild rodent plague areas are responsible for the endemic epidemic outbreaks in Iran Kenya Central Provinces in India Peru Brazil and elsewhere According to Baltazard the rat that has made the fortune of plague is not the original probably not even the actual proprietor of the disease but only the disseminator

The indispensability of the vector the flea in the rodent flea cycle is well known Only a comparatively few fleas feeding on an infected rodent with severe bacteremia become infected and fewer still become infective The numbers of fleas that become infective are conditioned by species feeding habits whether zoophilic (as are many wild rodent fleas) or anthropophilic and the efficiency as a transmitter which varies with species and is greatly influenced by climate Very important is the longevity of fleas as preservers or carry over agents from one season to another The climate and the size of the rat population control the density of the flea population in urban situations density is measured by the flea index the average number of fleas on each trapped rat A *cheopis* index of at least 3 appears to prevail during epizootics The most important and efficient vector of rat plague throughout the world is *Xenopsylla cheopis*

With a special apparatus for in vitro feeding (Kartman 1954) the plague vector efficiency of wild rodent fleas has been compared with that of *Xenopsylla cheopis* and none approached the high efficiency of this species The common squirrel flea (*Diamanus montanus*) is not very efficient In fact rodent fleas bite man only when they have been deprived of their usual host for some time and as a rule attack their own hosts or other rodents rather than man In wild rodent plague flea transmission from rodent to man apparently represents a weak link accounting for few transmissions to man Far more threatening is the introduction of plague by squirrel fleas into rat populations in rural and possibly urban areas (Meyer and Holdenried 1949) Avirulent *P pestis* can cause gastro-intestinal blockage in fleas and can be transmitted to mice by *X cheopis* and *X hawaiiensis* No enhancement of the virulence has been noted

Most investigators agree that primary pneumonic plague originates from a person ill with bubonic plague who has plague pneumonia of metastatic origin This first pneumonic case may then lead to others The patient may transmit the agent through droplets of sputum during coughing spells *P pestis* can be projected for several feet from the face of the patient when he coughs An enormous mass of bacilli in the lung overcrowding in badly ventilated buildings low temperature undesirable social habits and customs, such as kissing of the sick ignorance of precautionary measures against spread of infection—all these help bring plague to epidemic proportions

Control

Of course immunization against plague would not eradicate the disease Even though it could make man totally infection proof it would leave the disease in the reservoirs untouched and the threat perpetual

Systematic warfare against rodents is the fundamental operation in any completely effective antiplague program The really potent rodenticides—1080 (sodium fluoroacetate) and warfarin (Dicoumarin) (Pollitzer, 1954)—should be used by experienced persons to free cities of rats and to establish rodent free belts around towns and villages exposed to plague

In the control of epidemics the flea is a focal point of attack Remarkable success has been made possible by insecticides with residual action DDT (5% in kaolin powder) dusted in and around houses supplemented by treatment of clothing bedding furniture rat runs and harborages has been invariably effective In fact a threatening epidemic of bubonic plague was aborted in Haifa with this insecticide alone Latin American and French workers have had similar success

Treatment of epidemic plague victims can now be handled by mobile teams who treat patients at home or at local emergency hospitals and disinfest the house of fleas Therapy can be begun earlier and patients need not be transported for long distance thus reducing the danger of septicemia Physicians and nurses attending pneumonic or suspected pneumonic infections must wear hoods masks with goggles overalls and gloves Contacts and suspected contacts are first disinfected and segregated their temperatures are taken and chemoprophylaxis or chemotherapy is instituted The administration of sulfonamides (3 *Gm* per day for a week) appears to reduce the number of cases of pneumonic plague among people who are in contact with sufferers from plague capable of spreading the dis

of less than 10 millibars are the most favorable for checking it. The disease tends to occur during summer months in cooler climates and in the spring months in the hot dry climates of the subtropics, in tropical countries where the temperature is fairly constant throughout the year, the incidence follows the humidity curve. Many complex factors control the periodic waves, but fluctuations in endemic areas are always associated with variations in the climate. As the rodent population in urban areas is reduced by a rampant epizootic, and the fleas seek new hosts, persons of all ages and both sexes contract bubonic plague. General health, nutritional status and living conditions including overcrowding are determining factors in the mortality rates; there does not seem to be any racial immunity. Doctors and nurses who care for patients with pneumonic plague and other contacts, including funeral attendants, are liable to attack by the pneumonic form. In the rural plague regions men and children who handle or play with wild rodents are attacked more frequently.

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Recurrence of plague in commensal rats in countries where the principal natural reservoirs are squirrels or gerbils without notable repercussions in the nearby human populations is not uncommon. Plague in order to maintain itself in focal rural areas requires resistant wild rodents to survive epizootics. It is probable that they perpetuate the infection, and their fleas may spread it to commensal rodents. Plague cannot perpetuate itself without susceptible species capable of rekindling it. Complex ecologic factors in the habitual niches filled with hosts, parasites and

gen Cultivation at 37 C favors and accelerates dissociation Primary isolation from animal or human specimens is occasionally successful at only 22 C (Knapp 1956) Growth is optimal at pH 6 to 8 acidification favors R dissociation Most smooth to slimy light transparent colonies reach a diameter of 2 to 3 mm on the second day on agar medium containing serum or blood at 37° C the colonies are thin dry irregular with rough edges Shifts from S to R or intermediate types are largely influenced by high temperatures composition of the medium and age of the cultures Of the media used in the diagnosis of *Salmonella* the Endo medium is the most satisfactory Desoxycholate-citrate agar recommended for differentiation of *P pseudotuberculosis* from *P pestis* may suppress visible colony formation of the former (Knapp 1958 In broth growth is diffuse at 22° C clumped masses and occasionally ring and pellicle formation are seen The organism darkens meat and peptone by false pigmentation but does not color synthetic media It grows on gelatin without liquefaction but forms phosphatic crystals Some strains develop in thin yellowish brown layers on potatoes Five strains have grown readily in hydrolyzed gelatin or in basal amino acid medium without accessory growth factors Blood glucose and adequate aeration yield heavy cultures

Milk is not coagulated but alkalinizes slowly The following carbohydrates and alcohols are fermented and gas is not produced glucose maltose mannitol galactose arabinose glycerol isodulcitol levulose rhamnose trehalose and xylose Acid is not formed in amygdalin dulcitol erythritol inositol inulin lactose raffinose or saccharose Variations in fermentation of sorbitol salicin and dextrin both at 22 and 37 C have been described (Thal 1954 Knapp 1958) Cultures in peptone water containing 0 5 per cent glucose reach pH 4 6 to 4 8 at 37 C in 7 days but in the presence of 0 05 per cent glucose the final pH is 7 0 to 7 3 *P pseudotuberculosis* forms no indole and gives a positive methyl red reaction Nitrates and methylene blue are reduced rapidly A little H S is formed The catalase test is positive As urea is broken down alkali is formed

Antigenic Structure

Agglutination and reciprocal absorption tests demonstrate that *P pseudotuberculosis* has a group antigen common to a few isolated strains and an O antigen specific for the individual strains (Bhatnagar 1940 Thal 1956) The flagellar H antigen is common to all types The available strains have been subdivided into types I to IV with subtypes IA and IB Thal (1954) in a study of 119 strains recognized 5 types with 5 different O antigens Similar observations have been made by Knapp (1955) and by Girard and Chevalier (1955) but only groups I to III of Thal are identical with types I to III of Schutze A comparison of types IV and V with the type IV of Schutze cannot be made because the latter is no longer available However type IV of Thal differs not only with respect to the O but also the H antigen of all the strains thus far studied Consequently it cannot be identical with Schutze s type IV The studies are not exhaustive and it is reasonable to anticipate the finding of other types Most isolates from man and animals belong to type I

P pseudotuberculosis has the following antigens (1) 2 different thermolabile H antigens (2) 5 type specific thermostabile O antigens (3) strain pecific thermostabile O antigens of the subtypes IA IB IIA and IIB as determined by absorption agglutination tests on types I and II (4) at least 1 thermostabile antigen present in all types is common with an O antigen present in *P pestis* (Schutze 1929) Live cultures of avirulent strains produce a solid immunity not only against virulent strains of *P pseudotuberculosis* but also against *P pestis* in the guinea pig According to results of the Oudin double diffusion test in agar gel 5 of 7 antigens of *P pestis* are common to *P pseudotuberculosis* 2 are thermostabile and 3 are thermolabile The fact that phage lysis is not correlated with agglutination might be evidence that some strains of *P pseudotuberculosis* have more than one antigen in common with *P pestis* (*Gunnison et al 1951*) For the serodiagnosis of plague and for antigen analysis knowledge of the partial antigens between these two bacteria is of considerable practical importance (5) The antigenic relationship between *P pseudotuberculosis* type II and factor IV of the *Salmonella* B group and of *P pseudotuberculosis* type IV to factor IX of the *Salmonella* D group has been fully established

ease by expectoration The over all program for epidemic control requires the guidance of trained personnel—physicians entomologists mammalogists laboratory workers and sanitarians In this connection it is well to remember the words of Aubert Roche La civilization seule a detruit la peste en Europe seule elle l aneantira en Orient "

PSEUDOTUBERCULOSIS

PASTEURELLA PSEUDOTUBERCULOSIS

The large gram negative, elongated *P pseudotuberculosis* organisms are pleomorphic sometimes occurring in chains Bipolar staining is inconstant Motility lost when they are cultivated at 18° to 22° C is due to 1 or 2 parapolar, rarely 3 to 6 peritrichous flagella The organisms grow on medium containing bile salts or in amino acid solutions in the absence of accessory growth factors they produce no gas in carbohydrates no indole and litmus milk eventually becomes alkaline The antigenic relationship to the *Salmonella* suggests classification with the *Enterobacteriaceae* rather than the *Parvobacteriaceae* The term pseudotuberculosis must be reserved strictly for infections caused by *P pseudotuberculosis*

Human infections may take the form of an acute septicemia or the organisms may localize in the mesenteric lymph nodes of the ileocecal region under the clinical picture of acute appendicitis and gastro intestinal symptoms The recent addition of over a hundred infections of the latter type to the previous list of only 16 reported cases places pseudo tuberculosis in a more important position as an infection of man The means by which this recently recognized form is introduced is not known but the oral enteral route is suspected *P pseudotuberculosis* sporadically infects rodents pigeons turkeys and canaries In a few human infections it has been isolated from the blood stream during life and from the characteristic necrotic lesions at autopsy The mode of infection is not known

Synonyms *Streptobacillus pseudotuberculosis rodentium* (Preisz), *Bacterium pseudo tuberculosis rodentium* (Lehman and Neumann) Bacillus der Pseudotuberculose (Pfeiffer) *Bacillus parapestis* (Lerche) *Bacterium pseudotuberculosis rodentium* Preisz (Schutze) *Malleomyces pseudotuberculosis rodentium* (Pribaum), *Cillopasteurellosis* (Prevot) *Yersinia rodentium* (van Loghem)

History

The organism was first isolated by Malassez and Vignal in 1883 who inoculated guinea pigs with material from a subcutaneous tubercular lesion on the forearm of a child who had died of meningitis The animals developed nodules which, though histologically similar to those in tuberculosis, contained zoogleic masses of coccoid bacilli Many investigators described under various names a bacillus causing animal infections that seemed to be identical with the bacillus of Malassez and Vignal Comparative studies convinced Preisz that they were the same as those described by Pfeiffer The agent is being encountered oftener in epizootics among barnyard fowl cage birds cats and monkeys Because of its close antigenic relationship to *P pestis* various strains of the agent have been the subject of detailed biochemical and serologic study Recent papers stress its relationship to the enteric organisms and the incongruity of its inclusion in the genus *Pasteurella*

Morphology

P pseudotuberculosis varies in shape and size according to conditions of growth it may be coccal or ovoid under 1 μ long or it may form rods 0 5 μ by 1 5 to 5 0 μ with rounded ends either singly, in short chains or filaments It is gram negative but does not take bipolar stain as regularly as *P pestis* does At room temperature (from 18° to 22° C), even in repeated transfers, all typical strains are motile and swarm (Knapp 1956) Examination in the light and the electron microscopes demonstrates 1 or 2 mostly parapolarly located flagella 4 or 5 times longer than the bacterium Only single rods occasionally show peritrichous flagella Smooth and motile colonies develop best at temperatures between 20° and 30° C Neither spores nor definite capsules are formed though at 22° C a viscous layer (envelope) may be seen in India ink preparations

Cultivation and Biochemical Activities

P pseudotuberculosis grows on ordinary peptone broth when freely supplied with oxy

well fed adults Guinea pigs in particular are very susceptible mice and rabbits less so Parenteral introduction of pure cultures is fatal to guinea pigs which die in from 15 to 45 days revealing at autopsy local abscesses, enlarged regional lymph nodes with caseous centers and white gray spots studding the spleen the liver the lungs and bone marrow When bacilli are ingested small necrotic nodules appear in the Peyer s patches of the ileum and the cecum and there is caseous necrosis in the mesenteric lymph nodes and the omentum

In the course of epizootics guinea pigs may exhibit 1 of 3 types of clinical manifestations septicemia fatal in 24 to 48 hours classic pseudotuberculosis in which there is emaciation diarrhea and death in from 3 to 4 weeks and the glandular form with lymphadenopathy of the cervical and the thoracic nodes probably transmitted through bites At any one of these stages the bacillus may be present in the blood stream Severe septicemia is usually short and at autopsy there are acute splenic tumor severe hemorrhagic enteritis and accumulation of clear fluid in serous cavities Pathognomonic are the whitish nodules in the liver the spleen and occasionally in the lungs They represent focal necrosis—coagulated cells granular oxyphil debris and fragmenting polynuclear leukocytes frequently surrounded by foamy reticulum cells but rarely by epithelioid cells Giant cells are always absent The necrotic center may contain blood vessels plugged with bacterial emboli or remnants of vessels (Knapp and Masshoff 1954) Chronic lesions show extensive fibroblast and epithelioid proliferations that sometimes become granulomatous but never calcify Bacilli are numerous in such lesions

Immunity

Immunization against this infection has been of interest because of the active cross immunity with *P pestis* known long before the existence of common somatic antigens was discovered Chloroform killed heat killed or formalin killed suspensions of *P pseudotuberculosis* protect guinea pigs and rats against infection with *P pestis* The envelope antigen of the plague bacillus confers no immunity against *P pseudotuberculosis* Guinea pigs resistant to plague are still susceptible to pseudotuberculo is Antiplague sera usually agglutinate a variety of strains of *P pseudotuberculosis* to about the same titer but anti pseudotuberculosis sera do not react with any of the *P pestis* strains because they lack envelope antibodies (Bhatnagar 1940) Cross precipitation and cross complement fixation experiments with *P pestis* and *P pseudotuberculosis* have given contradictory results Anti plague sera do not confer passive immunity to guinea pigs against pseudotuberculosis

Guinea pigs that have recovered from the disease but possibly have a latent infection are immune to infection Active immunity may be produced in experimental animals through inoculation with living or killed cultures (Thal 1954 van Dorssen 1955 Sachdeva et al 1956) Failures must be attributed to use of immunogenically impotent strains In comparison with formalin killed organisms the vaccine made with living attenuated or avirulent strains has been superior (Thal 1954 Sachdeva et al 1956) A solid immunity of over 5 months duration has been induced with the avirulent type IV strain (32) against challenge with heterologous virulent strains The resistance is anti infectious and protects against infections with atoxic strains and subtoxic doses of cultures of toxic strains It does not protect against the exotoxin The antitoxic immunity produced with exotoxoid is effective against both the exo toxin and infection with toxic strains Old avirulent strains cannot induce immunity because of their inability to persist even temporarily in the tissues

Diagnosis

The disease cannot be distinguished either clinically or pathologically from typhoid paratyphoid tularemia or tuberculosis *P pseudotuberculosis* is usually isolated on ordinary medium without difficulty and in pure cultures from the blood during life or from pathologic lesions at death Differentiation from *P pestis* in rodent tissues or laboratory animals injected with plague suspect material and from *Salmonella* which produce no gas may be a matter of great difficulty Different media have been used but none is absolutely diagnostic Contradictory cross-reactions between plague and pseudotuberculosis bacilli may be due to antigenic variations as a result of temperature of incubation presence or absence of an envelope and other factors

and lends considerable support to the concept that the bacterium is related to the *Enterobacteriaceae* (6) Neither the view of Ransom that *P pseudotuberculosis* has no antigen related to *P pestis* nor that of Knapp (1956) that *P pseudotuberculosis* contains, with respect to agglutinability, a thermolabile, but with regard to agglutination complement fixation a thermostabile O antigen have been experimentally proved (7) Glycolipid antigens have not been isolated (8) Lysates and filtrates of 2 strains (New Orleans type III and Saranac type I) are lethal for mice guinea pigs and rabbits more sensitive to heat than similar preparations from plague bacilli they resemble endotoxins (Lazarus and Nozawa 1948 Girard 1950) Some strains of type III yield 2 types of toxin —an inadequately studied endotoxin and an exotoxin (Thal 1954) The thermolabile component easily converted into toxoid by formaldehyde is highly lethal for rabbits rats and mice and to lesser degree for guinea pigs Pathophysiologic effects of the pseudotuberculosis toxin are strikingly different from those of the murine plague toxin with respect to blood pressure changes hemoconcentration and local necrosis In in vivo and in vitro tests the pseudotuberculosis toxin has been neutralized by its antitoxin according to the law of multiple proportions A strain of *P pestis* phage lyses *P pseudotuberculosis* at 37° C but susceptibility decreased on successive transfer at 22° C due to the masking of somatic antigens This phage adapted to *P pseudotuberculosis* may lyse certain strains of Shiga Flexner Sonne and Schmitz dysentery bacilli Claims that *P pestis* spontaneously transmutates to *P pseudotuberculosis* have not been confirmed

Resistance

Recent tests indicate that suspensions of *P pseudotuberculosis* in saline are not regularly killed at 60° C in 3 hours they are inactivated within 5 to 10 minutes at 70° to 80 C One per cent phenol kills in 5 to 30 minutes formalin 5 to 10 minutes 0 001 per cent mercury bichloride or silver nitrate in 120 minutes and 60 per cent alcohol in ½ to 5 minutes (Knapp 1958) Drugs in the following concentrations stop multiplication neomycin 0 1 to 2 5 μgm/ml oxytetracycline chlortetracycline tetracycline HCl 0 25 to 2 5 chloramphenicol 0 5 to 6 25 streptomycin 1 25 to 10 penicillin 1 to 12 5 erythromycin, 125 to 500 sulfonamides 12 5 to 60 mg (Knapp 1955) Lyophilized cultures or cultures on blood medium in sealed tubes remain viable for years

Distribution and Range of Pathogenicity

Focal necrosis in the liver lymph nodes and the spleen invariably yield cultures of *P pseudotuberculosis* and in the early stages of infection bacilli are readily cultured from the blood stream Pseudotuberculosis as a fairly common epizootic and enzootic disease of animals has been reported from Europe sporadic infections have been observed in North and South America, England Japan and India

Infections among guinea pigs and turkeys are epizootic, while they are enzootic in rabbits and hares cats, chickens pigeons swans canaries sparrows, blackbirds monkeys sheep hogs horses lions, foxes goats and doubtless other untested animals The sources of 186 isolates (Thal 1954) were 93 strains from hares and rabbits 23 from beavers 19 from turkeys 10 from mink 8 from guinea pigs 6 from canaries 3 from monkeys 2 each from finches and paradise birds 1 each from rats cats foxes goats, deer pheasant partridge, pigeon, peacock and man 10 isolates were of unknown origin Serologically 126 strains belonged to type I 44 to type II 14 to type III and 1 each to type IV and V In France the isolates were distributed as follows 81 of type I 12 of type II 7 of type III 2 of type V (Goyon, 1956)

Many strains have apparent host preferences with varying pathogenic affinities similar to those of *P multocida* The experimental disease can be produced best by feeding guinea pigs rabbits and mice sparrows and canaries are quite susceptible white rats usually are refractory This organism has been recovered from soil dust water fodder and milk (Schutze 1929)

Pathogenesis

The organism probably is excreted by affected birds or rodents and can gain entrance to susceptible animals through any of several different portals As a rule the abdominal viscera are primarily diseased Breaks in the skin can also serve as portals Young inadequately fed animals are more susceptible than

ment of the regional lymph nodes They de scribed this as an abscess forming reticulocytic lymphadenitis The clinical and surgical findings were uniform An acute onset with temperatures of 100 to 104 F and pain in the middle or the right lower abdominal quadrant aroused the suspicion of appendicitis Laparotomy revealed a considerable amount of clear serous exudate and in most instances a normal looking appendix but the wall of the ileum and the cecum showed glassy rigid swelling due to infiltration In the mesentery particularly the ileocecal angle single or packets of enlarged lymph nodes were always present Knapp (1954) and Knapp and Masshoff (1954) identified the cause of this primary enteral complex to be *P pseudotuberculosis* Since 1954 Knapp has fully proved the nature of the infection either through isolation of the bacterium (13 lymph nodes 2 blood) or through serologic tests (94 cases) and histologic examination Histologic examinations have shown that the cecum and the appendix serve as portal of entry for the organisms (Graber and Knapp 1955 Riniker 1957) The carefully studied 109 cases leave no doubt that this form of pseudotuberculosis in children and young adults (2 to 23 years old) is by no means rare in Central Europe The course was usually benign after appendectomy The acute symptoms subsided rapidly without complication or antimicrobial therapy

A definitive diagnosis is assured by isolation of the organisms from the lymph nodes and the blood and by microscopic examination of the nodes In the sera of acutely ill patients antibodies have been detected by means of the agglutination test with live smooth variants of types I to V of *P pseudotuberculosis* (Knapp 1956) Titers of 1 80 to 1 12 800 have been recorded The titer does not fall until the infection has been completely removed from the me enteric nodes it may rise fall or remain stationary independently of clinical symptoms Complement fixing antibodies have been found less frequently and in lower titers (Knapp and Steuer 1956) Significant and subject to further studies are the observations that most patients sera contain no H agglutinins and boiled antigens though capable of absorbing the antibodies have been inagglutinable These observations contrast with the well known facts that serum prepared in rabbits or man by inoculation of carefully killed organisms contain H agglutinins and readily clump boiled antigens It is assumed that the O antigens may be thermolabile with respect to their ability to incite agglutinins and to become agglutinated but thermostabile in their ability to bind agglutinins (Knapp 1956) Until the causes for this different behavior have been explained it is obviously imperative to conduct the Widal test for pseudotuberculosis with live smooth cultures

In order to determine the prevalence of pseudotuberculosis in man such tests should be introduced into the battery of routine serologic tests in cases in which appendicitis is suspected or other intestinal disorders are not readily identified

TULAREMIA

BACTERIUM TULARENSE

Bacterium tularense causes a specific infectious disease of wild mammals and ancillary hosts in which it is maintained as a heterogeneous infection with insects acting as perennial reservoirs and vectors Man may enter into the chain accidentally or occupationally by contaminating his hands conjunctival sac or buccal cavity with the infected tissues or body fluids of certain animals especially wild rabbits and hares rodents birds or insects by the bite of an infected blood sucking fly or tick by ingestion of contaminated food or water or by inhalation of dust during the threshing of straw containing cadavers of infected rodents Tularemia in man is an acute moderately severe febrile disease with a tendency to pneumonic complications the clinical picture varies considerably according to the mode of entry of the agent [Synonyms *Pasteurella tularensis* (Bergey et al) *Brucella tularensis* (Topley and Wilson) and *Bacillus tularensis* (Kelser)] Recently a new species *Pasteurella novicida* has been isolated from water in the Western United States (Larson et al 1955) It is more closely related to *B tularense* than to the other *Pasteurella* but so far as is known it has not caused infection under natural conditions

HISTORY

Tularemia is not a new disease The adaptation of this parasite to rodent ectoparasites

Workers who have studied freshly isolated strains tentatively list the following (1) *P pseudotuberculosis* grows more rapidly and more luxuriantly than *P pestis* in transplants on artificial medium (2) The motility of *P pseudotuberculosis* must be tested after repeated transplants at 22° C —it is always absent after numerous transfers at 37° C (3) *P pseudotuberculosis* grown at 37° C dissociates frequently into rough variants, it acidifies medium containing rhamnose, glycerine and exclusively melibiose (4) Urea containing medium is broken down by *P pseudotuberculosis* but not by *P pestis* The polytrophic medium of Devignat and Boivin (1954) is useful (5) *P pestis* is virulent in the white rat *P pseudotuberculosis* is not (6) Polyvalent antiserum with antibodies against types I to V and type specific O serum as a rule agglutinate *P pseudotuberculosis* but not *P pestis* The sera of patients suffering from pseudotuberculosis may agglutinate bacilli isolated from the blood or tissues in dilutions of 1 80 to 1 10 240 (Knapp 1958) Infections caused by *P pestis* yield serum that agglutinates both organisms for serologic differential diagnosis the absorption of the serum is a prerequisite (7) A *P pestis* phage used in critical test dilutions at 20 C fails to lyse *P pseudotuberculosis* (Gunnison et al 1951)

Treatment

Little is known about the effect of antimicrobial drugs on pseudotuberculosis Two patients with the septicemic form recovered with sulfonamides and antibiotics (Snyder and Vogel 1953 Burianek et al 1949) 2 others died despite treatment (Hassig et al 1949) The appendiceal form requires no specific treatment recovery is uneventful In spontaneously infected primates sulfonamides had no apparent therapeutic effect

Epidemiology Epizootiology and Control

Of course the vast animal reservoir with its carriers and shedders is suspected as the source of human infections Single sporadic cases of the septicemic form have been attributed to contact with cats and to ingestion of infected meat (Moss and Battle 1941) or drinking water and contaminated articles of food (Knapp 1958) The disease does not spread from one person to another

Sporadic and epizootic outbreaks during cold wet weather attest to the importance of predisposing factors Nothing definite is known about the portal of entry or the mode of transmission among animals The nearly constant involvement of the abdominal organs incriminates the oral route Water or feed soiled with discharges from carriers or infected flesh from sick or dead animals are suspected Fleas cannot transmit the infection Field mice are suspected as biologic reservoirs

Pseudotuberculosis in Man

Until 1953 the medical literature of the world contained almost no references to this infection in man Bacteriologically proved were 16 cases (Sachdeva et al 1956) The clinical picture of 15 was dominated by a severe septicemic typhoidal course that ended fatally in 11 cases Vague prodromal malaise was followed by abrupt febrile onset with severe headache chills, general pains anorexia and occasional catarrhal symptoms The fever of the irregular or septic type at times reached 105° F Anorexia abdominal tenderness constipation or diarrhea and variable degrees of leukocytosis were usual Within a few days after onset the liver and the spleen became palpable and tender still other manifestations were septicemia, effusions into serous cavities bronchitis, pulmonary engorgement and edema and changes in the parenchyma of the liver the kidneys and the myocardium Death was usually preceded by icterus toxemia and stupor it occurred between the 10th and the 24th days but in 1 case not until early in the 3rd month

Diagnosis was established early by blood cultures or postmortem by culture of blood or organ specimens In some cases the diagnosis was supported by specific antibodies At autopsy the nodular caseous or abscessed necrotic foci from 1 to 10 mm in diameter, in the enlarged liver and spleen occasionally mesenteric lymph nodes and pancreas were pathognomonic

In contrast with the severe fatal form the more frequent infections with a benign course in children and young people already noted by Albrecht and Piechaud deserve attention Masshoff and Dolle (1953) reported an acute or subacute appendicitis occasionally accompanied by enteritis associated with enlarge

bacterium in appearance and virulence but attests to intrinsic homogeneity of the species (Traub et al, 1955) The optimal temperature for growth is 37° C and a narrow range of pH (7 6) Strong buffers are essential to counteract the tendency of *B tularense* to produce excessive ammonia as a result of amino acid metabolism

Glucose maltose and mannose are fermented without gas production fermentation of glycerol levulose and dextrin is irregular (Francis 1942) Glutaminase and asparaginase are present in all strains while only virulent strains degrade citrulline to CO NH₃ and ornithine (Fleming and Foshay 1955)

Antigenic Structure

Several strains studied in agglutination absorption tests by various workers appear to be antigenically uniform Francis and Evans found a serum prepared against *B tularense* agglutinated *Brucella melitensis* and *Br abortus* to about a quarter to a sixth of the original titer However neither organism absorbed the homologous agglutinins from the *tularense* serum *B tularense* was agglutinated to a low titer by antimelitensis and antiabortus sera but was unable to absorb the homologous agglutinins from these sera The heat labile antigen extracted from the plague bacillus does not precipitate with *tularense* antiserum but an antigenic extract of *B tularense* does precipitate to a low titer with an antiplague serum (Larson et al 1951) There is no cross immunity with *P pestis* or *P pseudotuberculosis*

Chemical analysis of the antigenic structure has furnished significant results Treatment with ethyl ether of an aqueous suspension of virulent or avirulent *B tularense* releases from the organisms an antigenic fraction not sedimentable by prolonged centrifugation at 4 500 rpm This soluble antigen is highly immunogenic in rats and mice (Larson 1945a Bell et al 1952) Purification by salt precipitation differential centrifugation and dialysis yields a product of uniform immunogenicity Electron micrographs have indicated that the product includes pure cell wall material Ouchterlony diffusion tests show at least 4 and possibly 6 antigenic components in contrast with the original aqueous supernatant of the extract which showed 9 precipitation zones Ultraviolet absorption tests showed no evidence of nucleic acid or protoplasmic contamination Lipid extraction of the soluble antigen yielded a polysaccharide and an amino acid complex containing organic phosphorus The serologic and in particular the immunogenic activity is very high from 38 to 77 micrograms immunized mice the degree of protection being proportional to the precipitation titer of the antigen (Ormsbee et al 1955 Ormsbee and Larson 1955) Several crude antigenic fractions have been obtained by phenol extraction of *B tularense* (Alexander 1950) described as the Boivin type antigen by Girard and Gallut (1951) or by acetone extraction of peptone broth cultures and considered as a protein or a protein carbohydrate fraction (Downs et al 1947) These antigens combine with erythrocytes and incite formation of specific antibodies The purified highly protective soluble antigen failed to stimulate agglutinin production in 16 to 42 day old mice but did so in 157 day old mice (Ormsbee et al 1955)

Differences in virulence of strains for various species of animal have been recognized Population changes occur through natural dissociation in liquid cultures the influence of microbial drugs or long continued residence of the organism in birds (Green 1943) Some strains of low virulence have been isolated directly from ticks Criteria to distinguish virulent from avirulent strains have been established by injecting animal species of varying degrees of susceptibility with *B tularense* and by calculating the number of organisms of a given strain necessary to kill each species (Owen et al 1955) A relationship between virulence and immunogenicity has not been established conclusively Cultures requiring 200 000 000 or more organisms for an LD_{50} as a rule are not smooth the organisms multiply little or not at all in the tissues of white mice and are not immunogenic It is surmised that some as yet unisolated antigen may be responsible for the ability of a strain to multiply and to immunize (Moody and Downs 1955 Downs and Moody 1955 Moody 1955) No significant differences in yield of antigen or protective activity have been found between virulent and relatively avirulent strains (Owen et al 1955) On the other hand the lower yield of antigen from the well studied avirulent strain 38 is attributable to its slower growth rate and the quantity of soluble cell wall antigens However the protection afforded per unit of antigen extracted

suggests that it is of considerable antiquity, probably long endemic in the Americas and Asia. G. W. McCoy in his studies on plague in 1910 discovered among ground squirrels (*Citellus beecheyi*) a disease characterized by pathologic lesions similar to those of plague. This disease was encountered in rodents shot or found dead in Tulare County, Calif., and McCoy and Chapin (1912) named the causative organism *Bacterium tularense*. Wherry and Lamb diagnosed the first infection in man and established the hare as an important source. Francis in 1919 and 1920 investigated rabbit fever in Utah and discovered that the blood of an infected rancher who had been bitten on the neck by a deer fly produced the same plaguelike disease in guinea pigs. In rapid succession he reported isolation of *Bacterium tularense* from jack rabbits, the transmission of the infection by the bites of the deer fly (*Chrysops discalis*) and rabbit louse (*Haemodipsus ventricosus*), the cultivation of the organism on a new medium and the means for serologic diagnosis. The importance of ticks as reservoirs and vectors has been elucidated by Parker and Spencer and by Calhoun and his co-workers (1954). Much of what is known about tularemia was learned through the extensive field, laboratory and clinical investigations of Francis and his colleagues in the U. S. Public Health Service. The cytotropism of the bacterium was first recognized by Francis, and its significance was established by Buddingh and Womack (1941). Outbreaks of murine origin and waterborne have assumed importance in Russia (Khatenever, 1943; Foshay, 1950) and the United States (Parker et al., 1951). Its spread as an epizootic from West Siberia through Southeast Russia to the dry climate areas of Northern, Central and Southern Europe has brought about extensive outbreaks.

Morphology

The shapes of *B. tularense* are diverse—large and small coccoid and bacillary, oval, minute, filamented, bean-shaped, dumbbell-shaped, bizarre and so-called "involution forms". Units less than 300 μ in diameter are filterable. It has an extremely delicate structure of very low electron density, which may account in part for its low survival rate when lyophilized (Shepard et al., 1955). It has no capsules or flagella and is not motile (Hesselbrock and Foshay, 1945). Dilute carbol fuchsin, gentian violet or polychromatic eosin methylene blue preparations will stain the bacterium in smears and sections.

Cultivation and Biochemical Activities

On semisolid medium such as gelatinized yolk of hens' eggs (McCoy and Chapin) or on glucose rabbit blood cystine agar (Francis), *Bacterium tularense* forms minute, transparent, droplike colonies that are mucoid and easily emulsified. Variations in colony types are claimed to be associated with differences in virulence and immunogenicity. Rough strains are avirulent, but colonial characteristics and electrokinetic cell surface reactions fail to distinguish virulent from avirulent smooth types or to assess the virulence of the virulent strains (Owen et al., 1955). Medium inoculated with infective tissue may show discrete growth in from 2 to 7 days, but in subcultures confluent growth appears in 24 to 48 hours. Routine blood cultures may be made on Rhamy's hemoglobin cystine agar or thioglycolate heart infusion agar. Fully virulent cells grow luxuriantly in consecutive transfers on a variety of media if the inocula are large. For large-scale cultivation, liquid medium composed of protein hydrolysates with extracts of blood cells are of value. Decreased oxygen tension or large inocula are required to initiate growth. Since *B. tularense* is cytotropic, it finds the best balanced environment within the cell of the host, either mammals or embryonated hens' eggs (Downs et al., 1947). It is highly infective but fastidious to cultivate because the specialized physical condition cannot be readily satisfied in artificial media. However, a pattern of 13 essential amino acids (arginine, aspartic acid, cysteine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, tyrosine and valine), glucose, sodium chloride, potassium phosphate, magnesium phosphate, thiamine, spermine and a suitable buffer system will grow certain strains from small inocula if a reducing thiol compound is added or semi-anaerobic conditions of growth are maintained. Thiamine is an absolute requirement of all strains tested. The constancy of nutritional behavior contrasts with the notorious variability of the

infected but no human infections have been traced to them

Certain species of ticks are important reservoirs *B tularense* has infected *Dermacentor andersoni* *D occidentalis* *D variabilis* *Ixodes ricinus* and *I californicus* Survival in at least 54 arthropods has been reported

The ticks directly related to human disease are the Rocky Mountain and the Western wood ticks the common Eastern dog tick the Lone Star tick (*Amblyomma americanum*) one species of deer fly (*Chrysops discalis*) and the mosquito (*Aedes cinereus*) In Russia *Ixodes ricinus* and *I persulcatus* are the natural reservoirs and *Dermacentor pictus* plays the same role in relation to voles as *Haemaphysalis leporis palustris* does to the American rabbit Argasid ticks (*Ornithodorus* sp) may be of importance in maintenance and distribution of *B tularense* among hosts in nature (Burgdorfer and Owen 1956) Body lice and their feces have remained infected for 53 days when kept at low humidity and temperature (Price 1954) *B tularense* is known to survive under many different environmental conditions in still water in streams (Parker et al 1951) moist soil hides infected carcasses and food One stream contamination in Montana persisted for 33 days Extensive water borne epidemics (Schmidt 1947) and sporadic cases due to handling fish or contaminated sewer water have been reported

Pathogenesis

According to Francis (1937) there are at least 20 known methods by which man can be infected with *B tularense* The most important are contact with infected vertebrates and discharges of arthropods accidental transmission through bites of mammals particularly carnivores or arthropods ingestion of partially cooked infected meat of vertebrates or of infected water inhalation of aerogenically dispersed material from cultures infected laboratory animals and fecal droplets of ticks

When the bacteria penetrate the skin or the mucosa there develops in about 10 per cent of the human infections a papular primary lesion that soon ulcerates The lesion usually appears on the hands the arms the face or in the conjunctiva but has been observed in the oral and pharyngeal cavities and in the nares The initial bacteremia usually lasts not longer than a week In highly susceptible experimental animals and in some patients the disease takes th form of septicemia from its onset or becomes severe causing death between the 4th and the 12th days From the site of inoculation the bacteria spread along the superficial and the deep lymphatics leading to dermal lymphangitic nodules lymphadenitis and bubo formation in more than 90 per cent of the human infections In the absence of discoverable primary lesions lymphangitic invasion is rare The transitory bacteremia gives ri e to diffusely scattered foci of necrosis in the spleen the liver the lungs lymph nodes bone marrow and possibly other tissues and organs With the appearance of antibodies bacteremia disappears but new lesions may develop by lymphatic extension A second and invariably fatal invasion may occur which usually disperses bacteria through both the systemic and the pulmonary circulatory systems resulting in the formation of miliary and submiliary foci of necrosis in nearly every organ

The typical primary ocular lesion is an ulcerated papule on the lower eyelid followed by general infection of the conjunctival sac characterized by congestion of the vesicle lacrimation damage to the eye and involvement of the lymphatics Ingestion of *B tularense* causes violent local reactions necrotizing pharyngitis abscesses in the roof of the mouth enlargement of the submaxillary and the cervical lymph nodes and ulcers hemorrhages and minute necrotic lesions in the gastro-intestinal tract There is always fever averaging 31 days in duration it usually rises initially falls and then rises again The sedimentation rate is elevated but the blood findings are not characteristic the leukocyte counts range from 5 000 to 20 000 with relative or absolute polynucleosis In the absence of ulceroglandular lesions the incidence of pneumonia is much greater than is generally thought Hilar enlargement due to involvement of the tracheobronchial lymph nodes with secondary extension into the parenchyma or primary involvement of the parenchyma without enlarged hilar lymph nodes with pleural fluid are revealed by direct roentgenogram Confluent lobular pneumonia similar to caseous tuberculous pneumonia followed by gangrene and abscess formation have been

from virulent or avirulent strains is always the same in the same species There are enzymatic differences between strains of graded virulence Highly virulent strains contain citrulline ureidase, while strains of low or no virulence had no such enzymatic activity (Fleming and Foshay 1955) Living virulent and avirulent strains injected intraperitoneally in doses of 10^9 organisms caused rapid death, while organisms killed by various means were not toxic (Moody and Downs 1955) The nature of this toxicity has not been clarified in fact the reports are contradictory No soluble toxin has been demonstrated by workers in the United States, but in Russia cultures on semisolid colloidal medium have yielded sterile filtrates that produce toxic reactions in mice Also it has been reported that *B tularense* contains an endotoxin The medium in which the organisms are lysed with dihydrostreptomycin contains endotoxin in accordance with the degree of lysis (Sakurai et al 1954) Suspensions of formalized bacteria contain a heat stabile antigenic fraction unrelated to virulence that causes characteristic dermal reactions in rabbits that could be neutralized with antiserum and partially inhibited by previous vaccination (Larson 1946) Treatment with ether removes this type of toxicity

RESISTANCE

This organism has survived in cultures for 22 years at 10° C without transfer in carcasses for 133 days in hides for 40 days in water for up to 3 months in pure glycerin at —14° C for 2 years and at —70° C for months Virulent organisms have survived in the muscle in refrigerated carcasses of infected rabbits for 4 months Dried in vacuo and held at room temperature it is viable for 4 years Heating at from 55 to 60° C for 10 minutes or cooking of infected tissues kills the organism Tricresol 1 per cent 0 1 per cent formalin 1 0 ppm of chlorine inactivate it 0 5 microgram per milliliter of methylene blue is inhibitive in vitro but not in vivo (Yaniv and Avi Dor 1952) The bacteriostatic level against 10^6 cells per ml in a suitable medium is 2 5 μ per ml for chlortetracycline and for chloramphenicol Complete lysis of the organism within 3 hours may be achieved with 16 mg per ml of dihydrostreptomycin (Sakurai et al, 1954)

DISTRIBUTION AND RANGE OF PATHOGENICITY

In man *B tularense* can be recovered during the first week of disease by inoculation of guinea pigs or by direct culture on suitable medium Pus taken from suppurating lymph nodes early in the disease contains viable bacteria, but later the lymph nodes do not At autopsy in man and in wild and laboratory animals, material from the acute necrotic lesions in the liver, the spleen, the lungs and bone marrow readily yields positive cultures Inoculation of guinea pigs with tissues from field mice occasionally has established the presence of the infective agent when there is no anatomically visible latent disease (Burroughs et al 1945) That *B tularense* infiltrates liver cells of infected mice and epithelial cells of the rectal sac and malpighian tubes of ticks and lice reflects its close adaptation to its hosts and explains in part the persistence of the bacilli in organs of mammals and transovarian transmission in ticks A quantitative study with uniform procedures (Downs et al, 1947) revealed the order of susceptibility to be mice hamsters guinea pigs, rabbits cotton rats monkeys rats dogs and 9 day old chicks Rats are 1 000 fold more resistant than white mice Even cold blooded animals are susceptible The naturally infected vertebrate hosts belong to the orders *Rodentia Insectivora Carnivora Ungulata* and the class *Aves*

Burroughs and his co workers (1945) listed 48 different species including the principal hosts the cottontails (*Sylvilagus floridanus*), jack rabbits and snowshoe rabbits (*Lepus* spp) In North America the cottontail rabbit is the direct source of over 70 per cent of all human cases of tularemia (Jellison and Parker 1945) Other animal sources are the gray squirrel fox squirrel, opossum woodchuck muskrat, skunk coyote fox cat, sheep deer water rat (*Arvicola terrestris* in Russia) vole and bull snake Epizootics among continental voles (18 species) have caused large explosive outbreaks in southern Russia Wild rats meadow mice several species of squirrel chipmunks lemmings calves and dogs have been found naturally infected but so far have not been known to cause human disease Game birds, quail prairie chickens pheasants and domestic chickens are among the avian sources Two species of grouse the sage hen and the horned owl have been found to be naturally

Considerable resistance has been produced in rabbits and rats (Larson 1945a Downs et al 1947) with killed-organism preparations but these or lysates protected only a small proportion of mice against as few as 2 to 5 LD_{50} of organisms A system involving the use of white mice for the study of induced immunity has now been devised by challenging the animals vaccinated subcutaneously with a strain fully virulent for mice but of diminished virulence for rabbits (Bell et al 1952) The vaccine that assured complete protection of 40 day old mice was the ether extracted vaccine of Larson or the purified antigen preparation derived from the cell wall (Larson et al 1954 Ormsbee and Larson 1955) It is believed that the dosage and the virulence of the challenge strain (425) is much higher than that ordinarily met with under natural conditions in human infections In the rodent disease the mortality rate is about 100 per cent whereas in the human disease the rate is 7 5 per cent in untreated cases Since the immunogenic fraction can immunize mice it may be justifiable to test it in human populations at risk of infection This view is not shared by Soviet scientists who are impressed by the superior immunizing properties of living strains of low virulence Mice vaccinated with sublethal doses of 19 to 24 strains of living *B tularense* have been protected against infections with at least 10 LD_{50} doses of virulent organisms (Downs and Woodward 1949) Certain living strains protected against at least 10 000 LD_{50} but when these strains were killed they protected against only 10 LD_{50}

The first human trial with a living avirulent strain prepared with the so called Gaisky avirulent culture induced local and general reactions in 72 per cent with prolonged moderate disability in some and fairly severe disability in a few cases (Kosmachevsky 1944) In a more recent effort avirulent strains have been selected and lyophilized (NIIEG) and the reconstituted vaccine is applied cutaneously by scarification Local skin reactions in the form of vesicles which later become pustules develop as local vaccine takes This vaccination reaction on the 12th to the 15th days may be accompanied by enlargement of the regional lymph nodes systemic reactions have been relatively few Allergic reactions develop in persons who have had tularemia or have been immunized successfully It is claimed that the protection afforded is from 90 to 96 per cent It lasts for at least 6 years and the use of avirulent living vaccine is part of the obligatory control of tularemia in the Soviet Union (Sil chenko 1955 Martinevski 1956)

Antiserum prepared in rabbits sheep goats or horses fails to protect mice or guinea pigs but the naturally resistant white rat can be protected with the polysaccharide precipitable antibody against 25 000 LD_{50} The results of serum therapy of man are comparable with those in the rat It has reduced mortality and shortened morbidity

DIAGNOSIS

Certain circumstances should lead one to suspect that a febrile illness is tularemia when the patient has killed skinned and cleaned rabbits or has been bitten by deer flies other arthropods or by a mammal in an endemic area In many cases however the mode of infection is obscure Clinical findings may suggest the diagnosis a febrile influenza like attack with initial severe fever a temporary remission and a further febrile bout of a fortnight s duration followed shortly by a local lesion possibly conjunctivitis and tender enlarged lymph nodes The pneumonic type particularly difficult to diagnose clinically is an atypical pneumonia

Intracutaneous injection of 0 05 ml of a specially prepared detoxified suspension of killed *B tularense* is a valuable diagnostic aid A tuberculin type of reaction appears within 48 hours It is almost always positive during the first week of disease when agglutinins are still absent Because sensitivity to the skin test may persist for many years after recovery from the disease it cannot be used as an index of recovery Intradermal injection of a minute quantity of antitularense serum causes an immediate specific erythematous edematous reaction

Specific antibodies are not demonstrable in the blood during the first 10 to 12 days The titer begins to rise in the 2nd week rises abruptly in the 3rd week and reaches its maximum in the 4th and 5th weeks A rise in titer is almost invariably conclusive proof of infection Since the agglutination test may be positive for the rest of the patient s life a positive test does not indicate that the disease in question is tularemia in the absence of symptoms

reported (Ivie 1955) Primary tularemic pneumonia has been reported in the clinical or epidemiologic literature Foshay (1950) has observed small areas of pneumonia in only 2 of 30 laboratory workers who contracted tularemia after exposure to infectious aerosols and dust containing American strains of maximal virulence The occurrence of pulmonary tularemia with or without the anginoid form has been described in the Russian literature

The gross lesions differ somewhat in different animals but they resemble very closely bubonic plague in the guinea pig The site of inoculation is necrotic the contiguous lymph nodes hemorrhagic and caseous the liver is covered with yellow gray necrotic foci and the spleen is studded with thick miliary necrotic granules Chronic tularemia in guinea pigs and squirrels is marked by enlargement of the lymph nodes and by necrotic foci resembling those in pseudotuberculosis Tularemia in rabbits gives rise to necrotic foci in the spleen and the liver the lymph nodes are free of lesions Findings are similar in mice in this species the spleen alone shows macroscopically visible nodules The relatively resistant white rat may have an enlarged spleen and few necrotic lesions At human autopsy tularemia lesions are generalized being found in the skin the conjunctiva the lymphatic structures and the tissues of the reticuloendothelial system there is necrosis in the spleen and the liver The lungs may be normal show discrete nodules extensive necrosis or large confluent areas of bronchopneumonia The heart seldom shows gross abnormalities the myocardium may be pale soft and flabby Fibrinous pericarditis thrombophlebitis fibrinous exudates in the peritoneum meningitis and encephalitis have been noted

The histogenesis of the typical lesions is characterized by a rapid accumulation of mononuclear wandering cells principally macrophages polymorphonuclear cells play little part in the reaction The fundamental cellular reaction depends on a complex biologic and physical relation between the bacteria and the host cells leading to necrosis in which there is an interplay of reticuloendothelial monocyte epithelioidal cells Vascular changes though conspicuous in the usual advanced lesions are not responsible for the necrosis nor is anoxemia (Lillie and Francis 1936) Of significance are the capacity of *B tularense* to multiply within hepatic and endothelial cells of guinea pigs and its selective affinity for thin ectodermal cells (Buddingh and Womack 1941) These characteristics certainly account for many features of acute and chronic tularemia

B tularense is a facultative intracellular parasite that may persist for years in the organs (Foshay and Mayer, 1936) Chronic relapsing tularemia and congenital transmission with death of the fetus during the 8th month of pregnancy have been reported (Foshay 1950) The damage to the host may be due to an endocrine, adrenal cortical disturbance in amino acid metabolism A pronounced inhibition of D amino acid oxydase has been observed in the liver and the kidneys of infected rats (Woodward et al 1954 Sbarra and Woodward 1955 Woodward and Mayhew 1956)

Immunity

An attack of tularemia confers immunity Butchers having suffered one attack are not known to experience others, despite probable frequent exposure Reinfection produces illnesses of varying severity from small local lesions and no constitutional reactions to acute febrile illness followed by mild prolonged systemic complaints (Green and Eigelsbach 1950)

The in vitro bactericidal activity of plasma is highest in the cow the dog and the sheep The lower activity in the guinea pig the monkey, the human the rabbit, the rat and the mouse (in the order listed) correlates to some degree with their susceptibility to experimental infection (Stanziale, 1957)

Periodic immunization has been recommended for exposed laboratory workers persons engaged in the wild rabbit industry or hunters of wild rabbits and in Russia for agricultural workers exposed to contaminated hay, grain and water The early studies were conducted on man Heat or formalin killed vaccine has provoked severe local and constitutional reactions Vaccines detoxified with sodium nitrite and acetic acid (Foshay) administered annually have not conferred complete resistance but the course of the disease was modified favorably Similar results were obtained with prophylactic injections of phenolized or acetone extracted vaccines (Kadull et al , 1950)

tact through infected blood sucking arthropods chiefly ticks biting flies even mosquitoes may cause any one of the 4 clinical types Direct or indirect contact with a reservoir host or an ectoparasite has not been established with any degree of certainty in over 50 per cent of the cases diagnosed in certain regions In infections contracted by heavily exposed laboratory workers who contract typhoidal tularemia it is usually difficult or impossible to determine the portal of entry The virulence of the invading strain may partly determine which clinical type will develop

Seasonal occurrence with its peaks in summer is associated with rabbit hunting and arthropod vector activity in the Western States Natural infection occurs in every month of the year but is most prevalent in the summer in the West There ticks are particularly dangerous from March to August deer flies from June to September Jack rabbits are hunted particularly from April to October and are a threat at that time In the East cottontail rabbits are hunted from November to January the months during which the human infections occur

Men and women of all races and all ages are susceptible Hunters farmers campers housewives butchers and laboratory workers are among those most commonly infected and the incidence reflects exposure The highest annual incidence on record is 261 cases in 1939 The mortality rate in 15 525 cases reported in the U S from 1915 to 1942 was 6 9 per cent (Francis) In 470 cases in Wisconsin between 1938 and 1947 the mortality rate was 4 per cent in 147 cases in Louisiana between 1948 and 1955 7 5 per cent The *lowering of the rate in recent years is attributable* to the use of streptomycin In 169 cases from 1934 to 1948 the rate was 33 per cent Despite the use of antimicrobial drugs the mortality rate in one series of 53 cases of typhoidal tularemia was 19 per cent In Russia the case fatality rate is 1 per cent

The key factors in the transmission are ticks lice fleas and mites these keep the infection alive in vertebrates belonging to the *Rodentia* (*Leporidae Muridae Sciuridae*) the *Canidae* and the *O idae* For its propagation in nature *B tularense* depends on the wood tick (*Dermacentor andersoni*) and the dog tick (*Dermacentor variabilis D occidentalis Haemaphysalis leporis palustris*) which feed on rabbits and other rodents The Lone Star tick (*Amblyomma americanum*) together with the dog tick are common hosts (Calhoun et al 1954) Of the many animals found infected in nature probably only a few serve as key reservoirs in any environment Human disease usually does not take place unless at least 1 per cent of the rodents in the region are infected

The chain of infection is efficiently maintained ticks in all stages from larvae to adults are transmitters adult females pass it to succeeding generations by transovarian transmission The multiple factors affecting the bionomics and the ecology of the insect and rodent reservoirs vary from region to region and from country to country creating a complex ecologic picture that has been sketched only in its broadest outlines Importing wild rabbits from regions of known endemicity into a tick ridden area creates new geographic reservoirs as experiences in Massachusetts have exemplified (Ayres and Feemster 1948)

Control

Endemic tularemia of rodents cannot be eradicated An increased incidence in human beings always coincides with an increase in the infected reservoir rodent population Under certain circumstances poisoning campaigns are indicated for example against water rats and mice in Russia Incidence probably would be reduced somewhat if interstate shipments of wild hares and their sale for food in markets and restaurants were supervised Until prophylactic vaccines are more widely *used sportsmen butchers and those who live* in regions where the infection prevails must be educated to the dangers of this disease To render meat of rabbits harmless thorough cooking is necessary Rubber gloves should be worn while dressing rabbits Drinking water from streams in endemic regions should be avoided Laboratory workers should be protected by vaccination face masks and rubber gloves Isolation is not necessary but discharges from suppurating local lesions must be disinfected

Early diagnosis and treatment with streptomycin reduces the case fatality rate

and signs (Ransmeier and Ewing, 1941) Agglutination reactions with *Brucella abortus* or *melitensis* occurring during tularemia are probably anamnestic responses due to the existence of subclinical or chronic brucellosis, rather than true cross reactions

Cultures should be made on dextrose cystine agar or thioglycolate blood agar guinea pigs mice or chick embryos should be inoculated with specimens of blood pleural effusions exudates from primary lesions or sputum from living patients or with heart blood taken at autopsy No effort should be spared to measure the virulence of the isolates by the newer methods (Owen et al, 1955) to provide the treating physician and the epidemiologist with needed information When agglutinins are absent, *B tularense* may be isolated from the nasal secretions and the sputum of persons suffering from tularemia who manifest no frank clinical signs of pulmonary involvement (Larson 1945c) A modified Ascoli thermoprecipitation test applied to tularemic materials is of value in early diagnosis and in rodent surveys (Larson, 1951)

Treatment

Streptomycin is bactericidal and is rapidly curative if administered in daily doses of 0 5 to 1 Gm It should be given for 5 to 8 days The broad spectrum antibiotics are also effective but less so than streptomycin The tetracyclines or chloramphenicol given orally in an initial dose of 25 mg /Kg of body weight and then in doses of 0 5 to 0 75 Gm every 6 to 8 hours are also recommended (Ransmeier et al 1949) The effect of the drugs is dramatic in acutely ill patients treated early in whom the disease is pursuing a stormy course The appearance of drug resistant mutants in human beings has not been recorded and any of these drugs is effective in controlling relapses

Epidemiology

Tularemia has been reported from many parts of the United States some areas are much more heavily infected than others There were from 11 to 25 cases per 100 000 population in Arkansas during the period 1937 to 1951 In 5 New England states only 20 cases have been observed since the discovery of the disease and in Vermont none at all In the U S between 1924 and 1950 there were 25 294 cases (Olsufev 1954), and between 1944 and 1955, the U S Public Health Service received reports of 10 865 cases Contact with cottontail rabbits was responsible for 65 5 per cent (Jellison and Parker, 1945) In California 81 per cent were attributed to contact with wild jack rabbits (Simons et al 1953) Tularemia has been reported from the U S , Alaska, Canada, Venezuela Mexico and Japan and since 1926 epizootic waves have spread from Siberia to Turkey and over most of Europe

It is an essentially sporadic disease It may become epidemic when a number of persons take an infected meal as in the cases reported by Amoss and Sprunt (1936) with 12 deaths in 20 victims, when contaminated drinking water is consumed (Schmidt, 1947 Parker et al 1951) or when deer flies are particularly infective, as when 30 of 170 residents of a boys camp were infected by bites (Hillman and Morgan 1937) No other infection has such a variety of modes of transmission, but human to human infection has not been recorded Numerous reports re emphasize the well known risk of laboratory infection

The sources of infection recognized in North America are (1) wild rabbits (rabbit fever) (2) terrestrial rodents (3) the biting fly (*Chrysops discalis*), (4) ticks (glandular tick fever) (5) semi aquatic mammals such as muskrats and beavers, (6) contaminated water (7) game birds and (8) sheep (Jellison and Kohls 1955) In the Soviet Union where rodents are the most important sources Khatenever (1943) listed the following types (1) trade outbreaks resulting from hunting skinning and preparing hides and carcasses of rabbits water rats and other wild game or fur bearing animals, (2) agricultural outbreaks from contact of farmers with mice during epizootics (3) contact outbreaks from food contaminated by infected mice (4) water outbreaks from direct contact with or ingestion of contaminated water but not including cases from contact with water animals (5) arthropod outbreaks The types may be interrelated the source and the mode of transmission are not always clearly discernible The most common clinical ulceroglandular or glandular types may be attributable to direct contact with infected animals for example in skinning or processing through a break in the skin or through conveyance of the organism into the conjunctival sac Intermediate con

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The Brucella

INTRODUCTION

The brucella are gram negative coccobacilli which are nonmotile and nonsporulating Capsules if present are small Some strains require added carbon dioxide for growth on laboratory media The organisms are classified into species on the basis of dye tolerances H S production and antigenic analysis The bacilli are strict parasites characterized by an intracellular existence in the host In animals such as cattle sheep pigs and goats genital and mammary gland infection is a common event and placentitis occurs with premature expulsion of the fetus These sites of localization are extremely rare in man The disease *in man is called brucellosi*

Synonyms Malta fever undulant fever Mediterranean gastric remittent fever Neapolitan disease Texas fever Rio Grande fever Bang s disease In animals Bang s disease infectious abortion epizootic abortion

HISTORY

Precise information concerning brucellosis began with the description of the clinical signs by Marston (1861) writing on gastric intermittent fever The isolation of *Br melitensis* type species of the genus and the etiologic agent of the disease as it occurred on Malta was made by Bruce (1887) who named the organism *Micrococcus melitensis* and obtained it from the spleens of human cases at necropsy As a result of the high incidence of this disease in military and naval personnel stationed on Malta the British government created the Mediterranean Fever Commission in 1904 The work of this group was rewarded by the isolation of the organism from the milk and the urine of apparently healthy goats as a sequel to the detection of brucellar agglutinins in their sera (Zammit 1905) The source of human infection was clarified at once and when the consumption of raw goat s milk was stopped the incidence of the disease among the service personnel dropped markedly

The second species was isolated by Bang (1897) from cattle suffering from contagious abortion and was named *Bac abortus*

The third species *Br suis* was isolated by Traum (1914) from the fetus of a sow

For many years the 3 organisms were regarded as unrelated until the close bacteriologic and serologic relationships between the abortus and the melitensis organisms were revealed by Evans (1918 1923) Then it was recommended by Feusier and Meyer (1920) that the 3 species be placed in a newly created genus *Brucella*

Important biotypes of *Br suis* were discovered which differed from the American suis strains strains of *Br abortus* from Southern Rhodesia were also shown to differ from the classic abortus strains in not requiring additional CO for growth

Additional strains have been isolated in recent years of which *Br ovis* (Buddle 1956) appears to be the only one with properties sufficiently distinct to warrant provisional species rank This species causes genital disease in sheep and is apparently widespread in New

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Zealand and Australia and it has been reported in California

After an extremely controversial period the pathogenicity of the 3 species for man was accepted, due in large measure to the studies of Huddleson (1945) on the speciation tests *Br abortus* differs widely from the other species in its ability to evoke clinical response after infection Epidemiologic and clinical data pointed to the fact that large numbers of *Br abortus* may be ingested over a long period of time via contaminated milk without producing severe illness in many of those exposed Under other circumstances especially via other routes of infection *Br abortus* may lead to severe *and even fatal infections*

An agent for the prevention and the control of the bovine form of the infection became available through the isolation of a strain of *Br abortus* by Buck in 1923 This strain was attenuated enough to prevent both its perpetuation in and its dissemination via milk samples from vaccinated animals Thus the safety of its use was assured although it is still virulent enough under certain conditions to cause human disease

MORPHOLOGY

The pleomorphism of these very small bacteria is a function of the age of the culture and the environmental conditions in the medium Although organisms are typically rod shaped it is none the less true that very short rods with pointed ends coccal forms and coccobacillary forms may predominate in a culture (The use of the generic term *Micrococcus* stemmed from the fact that the predominant form *in* Bruce s cultures was coccal) In one analysis of more than 300 strains it was shown that only 6 per cent of the *melitensis* strains and 46 per cent of the *abortus* strains were predominantly rod shaped The coccal form of *melitensis* will predominate in primary cultures from animal tissues or exudates whereas under the same conditions the bacillary form of *suis* and *abortus* will prevail The organisms are variable in length 0 3 μ to 2 3 μ They stain easily both when intracellular and extracellular but the former site requires much experience in differentiating the bacteria from the cell granules On the other hand the brucellae respond well to the Koster *and the* Macchiavello stainin methods for rickettsial and viral agents The alcohol fastness of the brucella may be added to the staining properties of differential value in the presence of other organisms when alkaline methylene blue containing 0 04 per cent KOH is applied for 1 minute washed off and replaced by 3 per cent aqueous safranin *for 15 to 20 seconds* after which the Brucella are blue while the background and non alcohol fast bacilli are red Capsules are present on freshly isolated smooth strains but have no relation to virulence of the strain While the brucellae are regarded as obligate aerobes, they will grow in the absence of oxygen when nitrate is provided They can obtain energy for growth from the oxidation of a variety of organic compounds such as sugars lactic acid glycerol and glutamic acid Their nutritional requirements are fairly complex since for good growth they need a variety of accessory factors in addition to the principal sources of carbon and nitrogen in the medium

Brucella abortus strains from most areas require upon primary isolation from cattle or humans from 5 to 10 per cent carbon dioxide in the gaseous environment whereas *Br suis* and *Br melitensis* will grow usually without this supplement However on occasion certain *strains of Br melitensis* are stimulated by added CO Although the major products of CO fixation in the case of *Br abortus* are pyrimidines there is no direct explanation of the CO requirement other than the suggestion that pyrimidine biosynthesis from CO may be more obligatory in those *Brucella* strains which require added CO than in the case for example of *E coli* which also fixes CO *but does not require* added CO (Newton Marr & Wilson 1954) Neither purines nor pyrimidines added to the culture medium obviate the requirement for added CO in the case of *Br abortus*

The *Brucella* grow well on laboratory *media* containing tryptose' or Albimi peptone or the more traditional liver and potato infusion Their primary isolation from animal or human host is not easy to accomplish due perhaps to the small numbers of brucellae in the peripheral blood many of which are in the polymorphonuclear neutrophils and small mononuclear leukocytes Attention must also *be given to the possibility of antibrucellar* toxicity of various peptones in the culture

media Schuhardt Rode Oglesby and Lankford (1950) have shown that toxic amounts of elemental sulfur may occur as a result of the degradation of sulfur containing amino acid in the peptones

A variety of synthetic media have been developed for the cultivation of different strains of *Brucella* One such medium proposed by Rode Oglesby and Schuhardt (1950) for the cultivation of *Br abortus* contains 18 amino acids in addition to 4 vitamins glucose and minerals The minimal needs of a CO_2-requiring strain of this pecies can be satisfied by considerably less complex media such as a mineral medium containing 4 vitamins and L glutamic acid as a sole source of carbon nitrogen and energy

An excellent medium capable of supporting the growth of several strains of *Br melitensis* was devised by Sanders Higuchi and Brewer (1953) This medium contains mineral salts *glucose 6 amino acids thiamine and nicotinic* acid By its use yields of over 125 billion cells per ml were consistently obtained with one strain

Certain sugars can be used as carbon sources for aerobic and anaerobic growth and can be slowly fermented by cells obtained und r aerobic conditions In fermentation tubes containing sugar media and inoculated with cultures of *Brucella* neither acid nor gas accumulates in quantities sufficient to make such tests useful for determinative purposes However these tests when performed by the method of Pickett (1955) provide a valuable adjunct in the study of atypical strains

All species of *Brucella* reduce nitrate to nitrite and *Br abortus* and *Br suis* carry the reduction further to nitrogen gas

H_2S is produced from sulfur containing amino acids by at least 2 of the species and occasionally by some strains of *Br melitensis*

All *Brucella* strains possess catalase and there is some evidence that a correlation may exist between the relative virulence of various strains within a species and the catalase contents of their cells Urea is decomposed by brucella but the quantitative urease activity of a strain does not aid in differentiating any one pecies from the other two (Sanders and Warner 1951)

The relationship between the accumulation of D alanine in the culture medium and the establishment of nonsmooth colonial mutants has been the subject of much study leading to the elucidation of mechanisms for the synthesis of this amino acid (Mika Braun Ciaccio and Goodlow 1954 Altenbern and Ginoza 1954) The essentiality of various metals for growth and maintenance of smooth forms has been shown by Waring et al 1953 and Evenson and Gerhardt 1955

The metabolism of glutamic acid by the 3 species strongly suggests that a urea cycle is operative in *Br abortus* and *Br melitensis* (Cameron and Meyer 1955)

CLASSIFICATION OF BRUCELLA SPECIES

At the present time the identification of an organi m as one of the species of brucella rests upon a series of tests (Huddleson 1943) some of which have consistently shown reliability others have been shown to be too strain specific that is they have succeeded mainly in further subdividing strains of a given species Among the latter and therefore of distinctly econdary importance are the morphologic appearance the crop yield in various media pigment formation crystal formation in the medium production of alkali in peptone water and the utilization of glucose The influence of an increased concentration of CO_2 on bacterial growth carries perhaps more weight when used to study cells obtained upon primary isolation from the host but the ability of certain strains of *Br abortus* to become independent of this additional CO_2 on further subculture lessens the significance of this property

The tests which have gained acceptance universally are

1 The production of H_2S The American varieties of *Br suis* and most *Br abortus* strains continue to produce H_2S for 3 or 4 days while growing on a suitable agar such as liver agar or Albimi agar medium *Br melitensis* and the Danish *Br suis* strains either produce none or minimal amounts

2 The use of liver Albimi or tryptose agar to which has been added thionin (1/30 000 and 1/60 000) basic fuchsin (1/25 000 and 1/50 000) methyl violet (1/50 000 and 1/100 000) and pyronin (1/100 000 and 1/200 000) allows one to distinguish in general the more typical strains of the 3 species as for example

A Thionin sensitive, insensitive to the other dyes (*Br abortus*)

B Thionin insensitive, sensitive to the other dyes (*Br suis*)

C Dye insensitive (*Br melitensis*)

3 Agglutinin absorption carried out with careful regard to the strength of the absorbing suspension and the dilution of the serum to be absorbed will yield monospecific sera able to differentiate between *Br abortus* and *Br suis* on the one hand and *Br melitensis* on the other

The existence of strains deviating from the standard reference strains of each species has stimulated several studies on atypical variants geographically peculiar in their incidence (for details and methods see Cruickshank and Madge 1954 Pickett and Nelson 1955 Renoux 1952) The results of several studies on large culture collections group the important strains as follows

1 *Br abortus* typical H S(+) thionin sensitive insensitive to basic fuchsin, methyl violet and pyronin Growth only or improved, in 10 per cent CO

2 *Br abortus* dye sensitive growth inhibited by all 4 dyes in the usual concentrations

3 *Br abortus* thionin resistant grows well without added CO (such strains common in Southern Rhodesia) also thionin *and* basic fuchsin resistant strains requiring CO for growth

4 *Br abortus* inagglutinable (Perhaps rough or mucoid)

5 *Br melitensis* typical No additional CO required insensitive to the 4 dyes agglutinated by monospecific antimelitensis serum H S(−) Strains producing H S for several days have been described

6 *Br melitensis* inagglutinable (Perhaps rough or mucoid)

7 *Br abortus* biochemically *Br melitensis* serologically mainly of bovine milk origin

8 *Br melitensis* biochemically *Br abortus* serologically mainly of human bovine caprine and ovine sources (designated *Br intermedia* by Renoux 1952)

9 *Br suis* American No additional CO required sensitive to basic fuchsin methyl violet and pyronin H S produced for 4 days agglutinated by monospecific antiabortus serum

10 *Br suis* Danish No additional CO required greater sensitivity in general to dyes and therefore tested in the presence of one half the concentrations given above Same differential dye sensitivity as American strains H S not produced agglutinated by monospecific antiabortus serum

11 *Br ovis* (n sp) typical additional CO required, sensitive to basic fuchsin (1/25 000) and methyl violet, H S(−) organisms have been observed only in non smooth phase are not agglutinated by antimelitensis or antiabortus sera which have been prepared against organisms in the smooth state Antisera prepared against a suspension of *Br melitensis* containing smooth and non smooth colonial phases agglutinate the ovine strains as well as rough or mucoid clones of *Br abortus* (Buddle 1956)

The differences between species are quantitative, and no single test distinguishes them For epidemiologic purposes the CO requirement, H S test dye tests and agglutination with monospecific serum will be satisfactory

The measure of susceptibility of the 3 species to chelation of necessary metallic ions by sodium diethyldithiocarbamate and the consequent effect on growth (Carbamate test) has been evaluated by Renoux (1952) and Cruickshank (1955) The test, again illustrative of the quantitative differences between species is limited in usefulness by the variability among the species in their response

The suggestion has been offered that the present species designations should be corrected by the adoption of a single species with appropriate varieties Thus Renoux (1952) proposed a new species, *Brucella brucei* with the several varieties melitensis abortus suis thomseni lisbonnei etc The increasing number of "atypical" strains which are being isolated and their interpretation in the light of modern concepts of bacterial genetics (Braun and Oglesby 1954) lends weight to the proposal of Renoux (1952) but it is unlikely that a change will be generally acceptable Objections to the acceptance of *Br ovis* (n sp) as a member of the genus have been raised (Meyer and Cameron 1956) on the basis of amino acid metabolism urease activity and carbohydrate fermentation However these tests require confirmation as to their legitimate value

RESISTANCE

Brucellae are very sensitive to direct sun light but are easily protected from ultraviolet wave lengths by the common vehicles in which they are discharged from the host The bacilli

in dead fetuses and fetal membranes will survive for as long as 6 months in pastures and barnyards In dry soil and in the absence of direct sunlight *Br melitensis* has survived for as long as 69 days At freezing or near freezing temperatures cells of a *Br abortus* strain survived over 824 days in the presence of bovine urine lake water tap water raw milk bovine feces and soil

During the aging process in cheese undergoing a lactic acid fermentation the brucellae in the contaminated source milk are killed within a few days in butter survival has been reported up to 11 days

Under experimental conditions the stability of the brucellae may be quite remarkable 45 per cent of collected organisms surviving on cotton from the aerosol state for 24 hours at 20 C and 12 per cent relative humidity The brucellae will survive 6 months or longer if the cell suspensions are added to ascorbic acid gelatin mixtures or dextrin solution

The brucellae begin to die off 10 minutes after being suspended in phosphate containing solutions and in many physiologic fluids (Ringer Locke Tyrode) On the other hand a mixture of inorganic salts and cysteine hydrochloride (ZøBell and ZøBell 1932) has provided excellent preservative qualities (less than 25% loss of viable cells in 7 days) and therefore is very useful to keep viable cell suspensions for further study

Destruction of the brucellae by pasteurization occurs easily even if the milk is heavily contaminated The brucellae are also quickly destroyed by the hypochlorites phenol formaldehyde and the quaternary nitrogen compounds area disinfection is accomplished readily with the use of such compounds as Roccal or Zephiran

MUTATION

Brucellae undergo population changes during growth on laboratory media producing colonies of the smooth intermediate mucoid and rough varieties Accompanying these colonial appearances are changes in antigenic structure and virulence Upon viewing colonies by means of oblique reflected light the changes in colony consistency can be visualized from the typically smooth transparent entire blue green tinted colony (S) to the rough type (R) showing increasing degrees of opacity and tinctorial changes to a brownish yellow (Henry 1933) Considerable assistance in determining the colonial properties has been afforded by the use of the 2 1 agar of Braun (1946) the crystal violet and 2 3 5 triphenyl tetrazolium dye tests (White and Wilson 1951 Huddleson Richardson Warner and Baltzer 1952) Antigenically nonsmooth cells are detected by their ready agglutinability in a 1 per cent solution of acriflavine performed as a slide agglutination test

The occurrence of atypical strains with the biochemical characters of *Br abortus* and the serologic properties of *Br melitensis* is most likely the result of mutation of the typical species Mutation rates for changes in dye sensitivities for example are of the order of $1\ 6 \times 10^{10}$ cells

Certain mutants compete successfully with the parent strain in the guinea pig The ability of colonial variants to establish themselves in the host in competition with the smooth types depends on the duration of the S type infection in the later periods of S type infection the hypersensitivity reactions fail to operate as efficiently against nonsmooth mutants consequently the ability of the latter to survive in competition with the smooth mutants in the tissues is improved (Berman Redfearn and Simon 1955) The fact that less virulent nonsmooth types are able to persist in localized abscesses may be a result of the protection afforded such organisms from the tissue sterilizing mechanisms or of the localized accumulation of metabolites in the abscess favoring the selection of mutants of greater degrees of resistance to these metabolites and giving the mutants a higher selective value The phenomenon of in vivo selection of mutants is among the most complex operationally because of the large number of variable affecting the host parasite relation (Waring Elberg Schneider and Green 1953 Braun 1956)

ANTIGENIC QUALITIES

Antisera prepared against the smooth form of the 3 species of brucellae agglutinate homologous and heterologous strains to equal titer If antiabortus or antisuis serum is absorbed with *Br melitensis* or on the other hand if antimelitensis serum is absorbed either with *Br abortus* or *Br suis* suspension a monospecific serum is produced which aggluti

nates only the homologous antigenic species (*Evans 1923*) *Such monspecific* sera can serve as diagnostic reagents It is not possible to separate *Br abortus* from *Br suis* by the usual serologic procedures

It is also known that antisera can be exhausted of all agglutinins by large doses of heterologous suspensions absorption by smaller doses leaves those agglutinins specific for the homologous suspension In studying the kinetics of the agglutination test Miles (1933) showed the existence of 2 optimal ratios indicating the existence of at least 2 antigens on the surface of the brucella cells

If a monospecific antimelitensis serum agglutinates only *Br melitensis* due to its "M antigen content and fails to agglutinate *Br abortus* which also possesses some M antigen it may be concluded that the M' antigen on the *Br abortus* cell is insufficient in amount or distribution for agglutination of the cells to occur even if fully sensitized Presumably the agglutination of *Br abortus* by an unabsorbed antimelitensis serum is due to the abortus system of antigens Miles (1939) has postulated that the brucella contain the 2 antigens 'A and M in different proportions characteristic of the 2 major antigenic species abortus and melitensis the abortus surface characterized by a distribution of A and M antigens in a ratio of 20 1 Quantitative precipitin reactions essentially confirmed these findings (Pennell and Huddleson 1938 Silverman and Elberg, 1950)

In addition to the major antigenic constituents of the smooth forms mucoid and rough variants possess antigens dominant to the respective phases However these do not interfere with the A and M antigens when the latter are dominant as in the smooth phase even though these so called r and z antigens (rough) eventually will induce antibody formation

Evidence exists that certain antigenic determinant groups may be identical in Pasteurella Salmonella Vibrio and Brucella which may lead to a certain amount of cross reaction when sera are being studied for etiologic identification These co reacting antibodies are readily identified by the agglutinin absorption test where the heterologous antigen will not absorb the antibodies for the homologous antigen

The antigens of brucellae which are obtained by phenol extraction at low temperature (Miles and Pirie, 1939) or by trichloracetic acid precipitation (Dubrovskaia, 1951 1954) contain phospholipids, carbohydrate and a formyl derivative of an amino polyhydroxy compound which may be an amino sugar The specific polysaccharides obtained by Dubrovskaia (1951) were composed of glucose, galactose, glucosamine and hexuronic acids but no evidence is as yet available to account for species specificity other than a suggestion of differences in amount of the above sugars

RANGE OF PATHOGENICITY

Brucellosis is primarily a disease of animals transmissible to man, for whom *Br melitensis* and *Br suis* are the most pathogenic according to the available evidence from epidemiologic studies, volunteer infections and laboratory infections

Br melitensis infections occur in goats sheep, catle, swine oxen and camel and probably in many other semidomesticated animal species The exact reservoir is somewhat peculiar to the geographic area in question reports of animal reservoirs show a broad infection spectrum In the goat the febrile period after deliberate infection begins within 3 to 4 days and the bacteremic phase occurs within the first week There are very few signs of the continuing infection which are regular enough to be of diagnostic use Abortion occurs in the primary infected pregnant animal but is not a regular accompaniment to pregnancy infection thereafter Organisms are shed in the milk and in vaginal secretions urine and feces Principal sites of bacterial localization are the lymph nodes the mammary glands the pregnant uterus and the kidney Sheep are somewhat less susceptible to infection and excrete organisms over a shorter period than goats but nonetheless constitute a major reservoir of melitensis infection in the epizootic form Strains of *Br melitensis* avirulent for man have been reported

Br suis infects swine, cattle, horses dogs fowl and hares but appears to be nonpathogenic for goats Silent infection can be acquired by weanling and suckling pigs Localization occurs in the reticuloendothelial system the uterus and the genitalia leading to abortion metritis orchitis epididymitis The Danish

strain of *Br suis* in contrast with the American strains does not appear to be pathogenic for humans

Br abortus occurs naturally in cattle swine goats horses dogs sheep fowl guinea pigs and rats In cattle the infection produces widespread necrosis and exudation in the placenta and uterine carunculae Death of the fetus results from interference with fetal circulation by the inflammatory process and abortion occurs after necrosis and separation of cotyledons Localization in the supramammary lymph nodes leads to excretion of organisms in the milk

The disease of sheep produced by *Br ovis* (n sp) is characterized by lesions of the epididymes the tunicae vaginales and the testes in the ram and by placentitis in the ewe with abortion or neonatal death of lambs

Experimental infections are easily induced in the guinea pig rabbit mouse monkey cotton rat and hamster The guinea pig is the most susceptible in terms of minimal numbers of bacilli required for infection The guinea pig eventually clears its tissues spontaneously Differentiation of brucellae species by lesion characteristics in the various animal species has been claimed to be a valid criterion but only the most expert comparative pathologist is qualified for such a taxonomically questionable procedure because of the great variation in virulence among strains of any of the species

Granted that the picture in the guinea pig varies with the species and with the dose injected one usually observes 4 to 5 weeks after subcutaneous injection swollen lymph nodes draining the injection site enlarged and engorged spleen showing a variable development of minute or large subsurface foci gray and firm in consistency and some scattered grayish foci in the liver Testicular lesions may be observed *Br melitensis* infections are most toxic for the guinea pig often leading to death The spleen and/or the lymph nodes often remain the only sites from which the organisms may be recovered in the guinea pig

PATHOGENESIS

The organisms infect man and animals via the skin the conjunctiva and the alimentary tract Under certain environmental conditions where the dried organisms have access to the air animal experiments suggest that the respiratory tract is al o of importance as a portal of entry Multiplication of the bacilli occurs on the mucous membranes at the various portals invasion of the regional lymphatics followed by entry into the blood classifies this group of organisms as following the hematohepatic route Selective localization occurs in the spleen the liver the lymph nodes bone marrow and the kidneys

Guinea pigs infected with *Br suis* by the inhalation of a single cell aerosol retain the organisms on the alveolar surface and allow bacterial multiplication to occur at that site in the first few days depending on th dosage employed for infection (Harper 1955) When the bacterial population reaches 300 times that required to induce a respiratory infection in half of the animals at risk then spread of the infection is detectable first to the cervical and the bronchial lymph nodes Soon afterwards dissemination throughout the organs takes place by the blood stream

The granulomatous reaction consisting of the accumulation of epithelioid cells and lymphocytes which may also include giant cells of the Langhans type and the foreign body type constitutes the basic tissue response to infection These granulomatous lesions are found in the lymphatics the liver the spleen bone marrow and other areas of localization and correspond to the response of the reticuloendothelial system to the phagocytosis which occurs in the vicinity of bacterial proliferation The genesis of this response in normal animals and its effect on the fate of the brucellae in the tissues has been studied extensively by Braude (1951) in the normal animal and in the solidly immune animal by Elberg Steiner and Doll (1955)

The natural relative susceptibilities of animal hosts and the correlative virulence of a strain can be gauged in part by the extent of necrosis and abscess formation in the guinea pig In these respects *Br suis* was characterized by a strong epithelioid and leukocytic response in contrast with the more benign purely epithelioid response induced by *Br abortus* (von Albertini and Lieberherr 1937 Braude 1951)

In acute cases elimination of brucellae through the glomerulus and the growth of the bacilli in the epithelium of Bowman s capsule and in the convoluted tubules accounts for cellular infiltration of renal tissue (Meyer 1943)

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Gorelick Pollack Braun and Riley 1951) The nonsterile immune phase is then succeeded by the phase of sterile immunity

At the present time the role of the various antibodies appearing in response to infection or successful vaccination is not clear Protective antibodies appear for example in the sera of mice after injection of brucellae These antibodies are capable of preventing death from subsequent injections of brucella in mucin but their role in immunity to infection is probably minor Certainly no positive correlation has been shown between immunity and antibody titer in terms of agglutinin precipitin opsonin or bactericidin Although serum from normal and infected humans and cattle is bactericidal for brucellae the serum of chronic brucellosis patients is inactive (Hall and Spink 1947) The reactivation of the serum by dilution has been attributed to the dilution of a specific inhibitor of the antibody complement system

In view of the predominantly intracellular location of brucellae in the host the mechanism by which bacterial multiplication is inhibited may ultimately reside in a specific cellular activity potentiated by a nonspecific serum system The protection from the antibody complement system and from antibiotics afforded by the phagocyte to the brucellae is well recognized Brucellae will exist in the circulating leukocytes of blood with a high concentration of antibody or antibiotic There is no doubt that immunity confers on the mononuclear phagocytes a significant ability to retard if not prevent bacterial multiplication in the presence of factors present or active only in hyperimmune sera The action of the serum factor enhances the ability of the immune mononuclear cell to resist destruction by ingested brucella The specificity of this cellular resistance is not strict as it is operative simultaneously against *Mycobacterium tuberculosis* as well as *Brucella* irrespective of whether tubercle bacilli or brucella cells are used to immunize the animals It would appear that the resistance of the phagocyte is nonspecific extending to those parasites which are primarily intracellular whereas the serum constituent necessary for the immune mononuclear cell to display its resistance may be induced by any antigenic material bacterial or otherwise (Elberg Schneider Fong 1957)

Hypersensitivity to the proteins and the polysaccharides of the organisms develops in the course of brucella infection and the Koch phenomenon of intensified elimination of brucellae by a previously infected animal has been unequivocally demonstrated (Redfearn Simon and Berman 1956) However the granuloma is not conditioned by the presence of hypersensitivity and the lesion seems to act to localize and destroy the bacilli a slow and often incomplete process

Artificial immunization of cattle has been successfully carried out especially in the United States using the live attenuated strain of *Br abortus* isolated by Buck in 1923 Immunization is applied in conjunction with controlled eradication of infected animals from the herd Successful immunization of goats has been accomplished with a living strain of *Br melitensis* derived as a reversion mutant of a streptomycin dependent strain of *Br melitensis* (Elberg and Faunce 1957) and a formalin killed preparation of *Br melitensis* in an adjuvant of mannide monooleate and mineral oil (Renoux Alton and Amarasinghe 1957)

An extensive program on immunization of humans has been reported using a derivative of the bovine vaccine strain called the BA strain for protection against *Br melitensis* in the USSR This live vaccine which apparently induces a major degree of cross immunity (*Br abortus* against *Br melitensis* infections) has been reported to decrease significantly the incidence of human infections among exposed occupational groups (Zdrodowski 1953)

CLINICAL PICTURE

The incubation period of the disease ranges from a few days to several months averaging between 10 and 30 days In the case of laboratory accidents with *Br suis* or *Br melitensis* the majority of cases have an incubation period between 4 and 8 weeks

The onset of the disease may either be abrupt with chills and fever or insidious and ill defined The most characteristic symptoms are chills weakness with fatigue and exhaustion severe frontal headache and backache At night profuse sweats and generalized aches occur especially in the joints the back of the neck and the lumbosacral area A fever

The brucella are intracytoplasmic during a large period of the infection selectively localizing in the mesenchymal and the ectodermal tissues (Meyer 1943) The presence of the organisms within macrophages fibroblasts, endothelial and reticular cells, interstitial cells of the testes and alveolar cells has been demonstrated in experimental animals by Castaneda (1947) and in a human case by Meyer (1943) Intracellular multiplication of the bacilli occurs in normal mononuclear phagocytes It is suppressed by immune mononuclear cells only in the presence of a still unidentified serum factor (Pomales Lebron and Stinebring, 1957 Elberg Schneider and Fong 1957)

The brucella endotoxin operates in the causation of illness in a manner similar to that of other gram negative endotoxins (Spink 1956) Clinical complications such as Herxheimer type of reaction resulting from too vigorous an application of antibiotics in brucellosis would appear to rest upon the same basis of endotoxin liberation as has been observed in certain other infections wherein tissues at autopsy of a fatal case are free of the etiologic agent isolated during the course of the illness The usefulness of the corticosteroids in managing these complications in brucellosis further justifies the attention given to the bacterial constituents and their ecologic importance

The reticuloendothelial system is the site of major tissue changes which are characterized as infectious granulomata The case fatality rate is variously judged to be 2 to 5 per cent Three types of fatal brucellosis have been described (1) a septicemic or relatively acute form (often superimposed upon another disease) (2) a focal or localized form (3) a chronic lymphogranulomatous type with a clinical course of several months duration

In addition to the lesions found in the reticuloendothelial system focal lesions of the endocardium involving both mitral and aortic valves have been recorded However bone localizations and affections of the central nervous system are two of the most important problems posed by the diseased host

The repeated showerings of the bacilli from the foci in the reticuloendothelial system into the blood help to explain both the great variety of inflamed tissues which harbor the bacilli as well as the incidence of the manifold signs and symptoms of hypersensitivity which play so important a role in the pathogenesis of the disease symptoms

RESISTANCE TO INFECTION

Epidemiologic data and experiments in human volunteers (Morales Otero, 1929) suggest that the susceptibility of man to *Br melitensis* infection ranges from 50 to 80 per cent Under severe exposure 40 per cent of those infected may exhibit frank and abortive clinical attacks The latent infection rate will be higher (75 to 80%) for this species as well as for *Br suis* exposure In the case of *Br abortus* infections, reports in the United States indicate that not more than 50 per cent of the human population is susceptible with a probable latency rate of about 58 per cent These figures are drawn from the natural outbreaks of the disease in limited population groups primarily acquiring the infection via ingestion of contaminated milk and might well be higher via other portals of entry *Br suis* appears to equal *Br melitensis* in causing not only high infection rates but also high incidence of clinical disease

Well recognized are the innate species resistance of the monkey to *Br abortus* and its high susceptibility to *Br suis* and *Br melitensis* the resistance of certain inbred families of rabbits and swine, and the self limiting nature of melitensis infection in cattle goats and sheep

The factors of age and sex are only partially defined up to the present time Immature members of susceptible species are more resistant to infection than the adults of the species with the exception of swine Epidemiologic studies in Minnesota on *268* cases of brucellosis placed the great majority of cases between the ages of 12 and 60 Children under 12 comprise a very small percentage of any infection group and unquestionably are more resistant in a relative sense However when the disease occurs in an undernourished and depleted population brucellae will attack all age groups and children respond as severely as adults

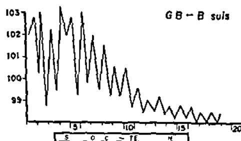
Immunity which develops as a sequel to natural human infection is also *relative* and evidence of reinfection in humans is available Guinea pig experiments show that *there is an* immunity to superinfection which requires 12 to 26 weeks for full development (Kelly

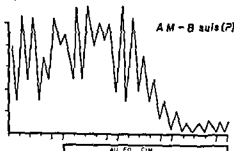
also be isolated from sternal marrow biopsy or aspirated material from lymph nodes and surgical specimens cerebrospinal fluid bile and urine Persistence in attempting to isolate brucellae from the blood is required and the study of many blood samples from a case is often more rewarding than dependence upon a particular moment on the febrile curve to take the blood specimen Incubation periods at 37° C using paired cultures one with added CO_2 for as long as 6 weeks with frequent subcultures onto brucella agar media are often required A particularly satisfactory method for blood culture is that of Tovar (1953) in which the colonies of brucella are encouraged to grow on the leukocyte layer of liquid blood cultures These are made in hermetically sealed jars containing citrated liver infusion or tryptose broth containing sodium bicarbonate to which 10 ml blood is added Castaneda (1953) has developed a system of rapid tests for bedside use for provisional diagnosis

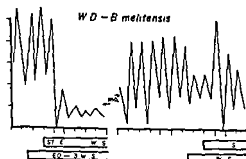
The agglutination test is the most reliable serodiagnostic procedure The complement fixation test is used in some areas but with diminishing return The tube agglutination test carried out on a patient s serum with a sufficiently broad range of dilutions (1/10 to 1/5 120) will reveal in the great majority of cases the agglutinating antibody When the test is studied with attention to the use of carefully standardized antigens and known positive serum obtained from state or World Health Organization reference laboratories much of the spurious difficulty formerly encountered disappears

In those cases with a strong presumptive clinical diagnosis of brucellosis despite a negative tube agglutination tests should be made for the presence of incomplete or blocking type antibodies This can be done by (1) performing the test in a diluent of 5 per cent sodium chloride instead of 0 85 per cent (2) using a protein diluent such as plasma albumin in place of saline (3) adding to all tubes in the series an equal volume of a dilution of positive serum which is known to agglutinate at that dilution (4) developing a positive test by addition of the Coombs re

Brucellosis Temperature Patterns with Treatment







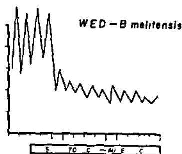


Figure 40

of 101 to 105° F may be reached stepwise during the first week of the disease, rising in the late afternoon and again in the early hours of the morning A drenching perspiration usually accompanies the drop in temperature Abdominal pain due to mesenteric adenitis or accompanying enlargement of the liver or the spleen occurs frequently Anorexia is very frequent and nervousness or mental depression is significant in incidence There may be constipation or diarrhea

The most frequent and distinctive signs of brucellosis are fever of a diurnal type, lymph adenopathy accompanying splenomegaly in the more seriously ill and hepatomegaly A retention jaundice is often overlooked

The hematogenous spread is probably the basis for localizations on the heart valves with resulting subacute endocarditis destruction of the intervertebral disk and adjacent vertebral bodies encephalitis meningitis encephalomeningitis peripheral neuritis radiculoneuritis with spondylitis hepatitis angiocholitis and cholecystitis This baffling puzzle in differential diagnosis has been emphasized by the cataloguing of more than 150 different symptoms Personality changes and other signs gathered under the term 'neurobrucellosis' confuse the diagnosis of older cases with their genuine anxiety states tensions and general apprehensiveness solidly based on a concept of repeated endotoxin assault Even abortion may occur, albeit rarely, in the human host

The average duration of the illness is from 3 to 4 months, although the sense of well being in the presence of bacteremia durin certain therapeutic regimens may require adjustment of this figure In the absence of early therapy an ill defined 'chronic disease may ensue without typical symptomatology For further details on the clinical picture see the monographs by Harris (1950) and Spink (1956)

Figure 39 from the data of Cluff, Trever and Peeler (1957), illustrates the various fever curves in untreated infection Figure 40 shows the effects of treatment with streptomycin and/or tetracycline and points out the difficulty in understanding therapeutic influences on the fever curve

LABORATORY TESTS

Isolation and identification of the organisms constitutes the only definitive test for brucellosis Blood is the most frequently positive specimen but on occasion the organisms may

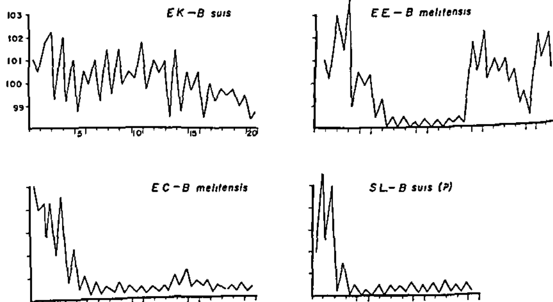


Figure 39

FIGURE 42

with virulent strains The difficulty with the carbohydrate protein nucleoprotein and purified preparations used for skin testing is that they usually induce antibody formation The polypeptidelike preparations when adequately purified are not antigenic in this sense are active in very high dilution and reveal the sensitive state very early in the course of the infection (cf Morales Otero and Gonzalez 1939 Huddleson 1943 Fust Loffler Mosimann and Schock 1949 Benedict and Elberg 1953) The highly active preparation of Ottosen and Plum (1949) is especially useful in this regard and simple to prepare

TREATMENT

The tetracyclines streptomycin or dihydro *streptomycin in combination among them* selves or with sulfadiazine have led to striking symptomatic relief in from a few hours to 3 or 4 days after initiating treatment It has been recommended to give 0 5 Gm of either streptomycin or dihydrostreptomycin intra muscularly twice daily for 2 weeks and 0 5 Gm sulfadiazine orally 4 times daily for 3 weeks The superiority of the combinations of tetracyclines with streptomycin has been

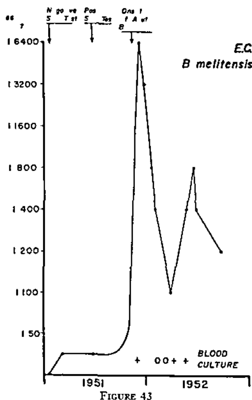


FIGURE 43

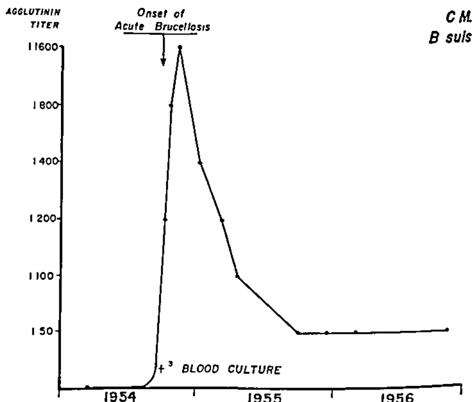
agent (antihuman globulin serum) to the non agglutinating preparations or (5) centrifuging all tubes before making the reading

Since a "positive" serum is a matter of titer determination attention must also be paid to the level of agglutinins in the population of the area in general Usually, titers of 1 160 and up are indicative of infection and more respect is accorded to sera the titers of which are in the higher ranges

The serologic pattern in a group of 60 cases of acute brucellosis occurring in an ideal diagnostic situation has been studied carefully by Cluff Trever and Peeler (1957) Three distinct patterns of antibody response occurred in these cases of *Br suis* and *melitensis* infections which are illustrated in the following 3 figures supplied by the authors

One pattern shows an abrupt rise in titer with the acute infection followed by a fall to below 1 100 (Fig 41) The second pattern shows a rising titer with the development of acute disease and a maintenance of the titer (over 1 100) during subsequent years (Fig 42) Finally, in Figure 43 is shown the type which has multiple elevations with intervening periods of depressed titer, the secondary elevations often accompanied by symptomatic relapse

The skin test in which 0 1 ml reagent is injected intracutaneously, provides information in 24 to 72 hours on the allergic state of the skin or the mucous membranes depending upon its site of introduction A positive reaction in brucellosis like a positive tuberculin reaction indicates that the reactive host has had at some time an experience with the antigenic components of the brucellae A positive reaction cannot serve on its own as an indicator of the current status of infection in humans or animals It may be of use in classifying herds of animals as having had experience with brucella and in such cases may overcome the difficulties of distinguishing agglutinin titers arising as a result of immunization from those caused by infection



Figs 41 to 43 Patterns of agglutination titer observed in brucella infections with reference especially to relation between clinical onset bacteremia induction of allergic response and titer

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striking In such cases the cyclines are given orally 4 times daily for 21 days with the above dose of dihydrostreptomycin or streptomycin A discussion of the rationale for the various therapeutic regimens is presented by Knight (1950) and Spink (1956)

The protracted natural course of the disease makes it difficult to appraise the various procedures for clinical and serologic relapses occur The specific immune system also affects the outcome of treatment and may well repay therapeutic conservatism in the early days of the infection

Adrenal steroid therapy has been ably studied by Spink and colleagues who have achieved excellent results through the careful use of the hormones simultaneously with the antibiotics on properly selected cases

Specific brucella products are also in use in various areas Considering the high degree of hypersensitivity and the symptoms resulting therefrom in those for whom this type of therapy is especially proposed the use of antigens in therapy has much theoretical justification The reports on the nonspecificity of the materials employed provoke questions concerning the mechanism of clinical success achieved (Harris 1950 Visani 1952)

EPIDEMIOLOGY

The Brucella are transmitted to man from the natural reservoirs in domestic animals The growing number of natural hosts indicates that an active interchange of the various species among animal hosts is occurring Data on insect transmission and the isolation of Brucella from wild rodents suggests secondary cycles which may finally reveal the complete natural history of human and animal infection Transmission to man occurs by contact ingestion, accidental inoculation and inhalation Contact with infected animals or their tissues blood aborted fetuses placenta urine etc allows brucellae to invade microscopic abrasions of the skin and the mucous membranes Ingestion of contaminated milk cream cheese or other dairy products made from raw milk containing brucellae is the second major route of transmission Pickled meats and other uncooked foods contaminated by excretions of infected animals have also been incriminated Studies of laboratory acquired infections of humans as well as studies on infection of animals via the respiratory route suggest that inhalation infection of humans should be considered in the absence of strong evidence for the other routes mentioned

Brucellosis is a significant occupational hazard among veterinarians packing house and rendering plant workers, animal production farmers, butchers, processors, stock buyers stock handlers and laboratory workers

No accurate figures for the incidence of brucellosis are available Annual estimates for the United States remain around 4 000 to 8,000 new cases per annum but this is very approximate Only in Denmark has the disease truly been eradicated With the end of World War II the control program in the United States is again proving to be effective in reducing the numbers of infected herds and this will inevitably affect the incidence of human cases

In humans the incidence in males is considerably higher than in females in groups which are in contact with livestock However the incidence is more equal in the two sexes where infection is due to use of unpasteurized dairy products, indicating that the difference is not due to unequal susceptibility of the sexes but to opportunity for exposure (Hardy et al 1931)

CONTROL

It is a principle among most of the western countries that the problem of controlling human infections will be solved with the control of the infection in the animal reservoir Measures to accomplish this are based upon regulations governing interstate movement of infected animals, a program designed to immunize young animals, the segregation and/or the slaughter of serologically positive animals improvement of environmental sanitation and education of the public concerning the disease To all of this may be added shortly the human immunization program which in many areas of the world may constitute the only economically effective way of handling the problem when the economic losses contingent upon the control in animals are too heavy to bear A start in this direction has been made in Russia from which encouraging reports have been published in the *Soviet Journal of Microbiology Epidemiology and Immunobiology* during the past several years

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20

Listeria and Erysipelothrix

Listeria and *Erysipelothrix* are gram positive rod shaped nonsporulating aerobic bacteria of the family *Corynebacteriaceae* which which are pathogenic for a wide variety of mammals and birds While the disease entities produced by the two organisms are not similar the two appear to be related taxonomically Some authorities place them in the same genus although there is considerable disagreement as to the validity of this

LISTERIA MONOCYTOGENES

History

Listeria monocytogenes is associated with sporadic cases of meningitis and granulomatosis infantiseptica in man and with a number of clinical syndromes in animals and birds The organism was first isolated by Murray Webb and Swann (1926) from an epizootic among stock rabbits and guinea pigs and described by them under the name *Bacterium monocytogenes* in view of the mononuclear leukocytosis observed in these animals Pirie isolated an identical organism in 1927 from an epizootic of wild rodents in South Africa and created the generic name *Listerella* Later this was shown to be a homonym and the name *Listeria* was proposed by Pirie (1940) It seems probable that the same organism was isolated from the spinal fluid of patients with meningitis much earlier by Atkinson in 1917 and Dick in 1920 In recent years there has been a renewal of interest in both human and animal infections with *L monocytogenes* which has been found to have a very wide spread geographic distribution There is a much greater awareness of the possibility of such infections on the part of physicians veterinarians and bacteriologists alike A recent monograph by Seeliger (1955) lists 350 references to *Listeria* from the newer literature

Morphology and Cultivation

L monocytogenes is a gram positive facultatively anaerobic rod approximately 0 5 micron in width by 1 to 3 microns in length The organism occurs in both a smooth and a rough form Small rods with rounded ends showing palisade formation and short chains predominate in the smooth phase Metachromatic granules are never observed Young cultures in the rough phase consist almost entirely of filaments up to 60 microns in length while older cultures contain many pleomorphic forms Young broth cultures show a characteristic sluggish tumbling end over end motility Cultures grown at 37 C contain both nonflagellated and monotrichous organisms while those grown at room temperature are peritrichous with up to 4 flagellae

The organism grows scantily to well on simple media growth is improved markedly by the addition of glucose ascitic fluid or blood Colonies in the smooth phase of nutrient agar are up to 0 8 mm in diameter after 24 hours at 37 C and are almost transparent by transmitted light Those in the rough phase are slightly larger with a granular center Both smooth and rough phase colonies may be up to 2 mm in diameter on horse rabbit

or human blood agar and most strains show a narrow zone of beta hemolysis On tellurite medium colonies are black, smooth, circular and 0 25 to 0 5 mm in diameter after 48 hours No growth occurs on MacConkey's agar Good growth occurs in infusion broth in which the smooth phase produces an even turbidity while the rough form gives a thread like granular growth which does not disperse readily

The biochemical reactions of *L monocyto genes* are somewhat variable Production of acid without gas in 24 hours from glucose rhamnose and salicin is most constant and reliable (Barber 1939 Julianelle 1941 Reed 1954)

Resistance

Listeria is less susceptible to heat than many pathogens, since it has been shown to survive 5 minutes at 80 C (Murray 1955) Carefully controlled pasteurization of milk containing more than 1 000 bacteria per ml does not effectively destroy *Listeria* (Girard, 1957) *Listeria* will survive without loss of virulence for 3 to 4 years at 4° C either on culture media (Murray 1955) or in brain suspensions (Gray et al 1948) It will re main viable and virulent after 8 weeks in 20 per cent NaCl at 40° C but survival is very short in normal saline or distilled water *Listeria* added to food pellets straw and wood shavings will survive up to 26 weeks in the dry state and there is indirect evidence that it persists for at least 2 weeks in animal pens and bedding straw (Gray et al 1956)

The organism resembles most pathogenic bacteria in its susceptibility to the chemical agents ordinarily used as disinfectants Shimizu et al (1954) have shown that *Lis teria* will survive exposure to 1/10 000 guano furacin and they have incorporated this into a differential media for isolation of the or ganism Girard (1957) uses furacin for the same purpose with variable results depending on the source of the specimen from which *Listeria* is to be isolated

Antigenic Structure

Agglutination and agglutinin absorption technics have been used extensively to study the antigenic structure of *L monocytogenes* Paterson (1940) has recognized 4 serotypes of *L monocytogenes* based on the H anti gens The O antigens are distinctive for types 3 and 4 but identical in types 1 and 2 It appears that types 1 and 4 are world wide in distribution but type 3 has been reported only from Denmark and Eastern Germany while type 2 occurs mainly in Great Britain Biotypes are recognized within serotypes 1 and 4 based on their ability or lack of ability to ferment melizitose According to Seeliger (1955) the majority of type 4 strains are positive and type 1 are negative There ap pear to be some antigenic components of *Listeria* which are common to other pathogens particularly *Staphylococcus pyogenes* and *Streptococcus faecalis* (Seeliger and Sulz bacher 1956) Although *L monocytogenes* contains a nonantigenic lipid that enhances antibody formation in animals immunized with other bacteria (Stanley, 1949), the immune response of infected animals is often very poor Osebold and Sawyer (1955) have demonstrated that sheep can be protected against large challenge doses of *Listeria* by prior subcutaneous inoculation of live viru lent culture

Distribution and Range of Pathogenicity

Spontaneous infection of a wide variety of animal species including man has been re ported from many parts of the world *L monocytogenes* has been isolated and identi fied from at least 27 species rabbit hare mouse rat hamster, chinchilla guinea pig gerbille lemming vole sheep, goat cattle swine horse fox dog ferret raccoon chicken canary duck, goose, snowy owl and man Undoubtedly other species will be involved as more detailed investigations are carried on The majority of infections have been proved in domestic or captive animals but it is significant that there exists an abundant reservoir among wild species some of which are predatory and many of which are migra tory *L monocytogenes* has been reported from nearly 30 countries ranging from the Arctic to the tropics The increasing number of human cases and the fact that it is being associated increasingly with disease of live stock and poultry makes listeriosis a much more important disease than it has been con sidered heretofore

Pathogenesis and Symptomatology

L monocytogenes infections produce many different symptoms depending on the animal species and the portal of entry

In cattle sheep and goats meningo encephalitis is a prominent form occurring in the brain stem with frequently a flaccid paralysis of various muscle groups The lesions may extend to the spinal cord with resulting unilateral ataxia The head and the neck are turned to one side and the animal tends to move in circles hence the common name of circling disease Septicemia and monocytosis are uncommon in ruminants A generalized infection occurs in rabbits and guinea pigs with focal necrosis particularly in the liver and the suprarenals and usually the disea e is accompanied by a marked mono cytosis The mononuclear response in rabbits has been studied intensively and a serologically inactive lipid has been extracted from *Listeria* which is capable of producing mono cytosis in rabbits These studies have been confirmed and extended by Girard and Murray (1954) The natural disease in rabbits is accompanied by marked edema and extensive serous exudates while in guinea pigs there is usually massive myocardial necrosis In the dog and the fox listeriosis has been described as a distemperlike di ease In some animals the disease appears to be a generalized infection which in the lemming at least is not accompanied by any obvious lesions although it is fatal

Abortion due to *Listeria* has been described in sheep goats swine rabbits and cattle In many instances the fetus showed an extensive necrosis very similar to that seen in human cases in newborn infants In some of these cases the organism could be isolated from most parts of the aborted fetus and sometimes from the placenta membranes as well as from the vaginal discharge of the female In the chicken listeriosis is a septicemic disease often accompanied by massive myocardial degeneration and necrosis A rather peculiar lesion in animals is keratoconjunctivitis which can be produced experimentally in rabbits guinea pigs and hamsters This reaction is produced only by *Listeria monocytogenes*

Human listeriosis is said to be a rare disease and most reviews list only some 40 cases However recent reports from Germany list 150 bacteriologically proved cases (Seeliger 1955 Krepler and Flamm 1956) Earlier cases of meningitis mononucleosis and granulomatosis have been reviewed and 36 dating from 1891 have been suspected of being listeriosis It is highly probable that human listeriosis is much more common than is realized since diagnosis depends on isolation and identification of the organism which is not always easy The various types of human listeriosis which have been described fall approximately into the following incidence of categories 33 per cent meningitis or encephalitis 29 per cent granulomatosis 21 per cent septicemia 8 per cent mononucleosis 6 per cent conjunctivitis (Murray, 1955) Clinically *Listeria* meningitis in man resembles other purulent meningitides and the type of cellular response in the spinal fluid is mononuclear or polymorphonuclear in nature Septicemic cases occur under a variety of circumstances Following the report of Nyfeldt (1929) of the isolation of *Listeria* from blood or spinal fluid of patients with infectious mononucleosis considerable interest developed regarding the possible etiologic role of *Listeria* in this disease Other workers have frequently failed to obtain positive cultures and there is no conclusive evidence at present that *Listeria* plays a part in infectious mononucleosis (Girard and Murray 1951 Seeliger 1955 Krepler and Flamm 1956)

A form of human listeriosis known as granulomatosis infantiseptica is second only in importance to listerial meningitis This is an intra uterine infection with a high mortality for the fetus or the newborn child The disease is a generalized infection characterized by extensive focal necrosis (Fig 44) esp cially of the liver and the spleen more rarely of the lungs and the intestines There may also be meningitis in some cases Potel (1951) and Reiss Potel and Krebs (1951) have studied over 40 cases of granulomatosis infantiseptica in the region of Halle in Germany Patočka et al (1953) have reported 10 cases in the region of Prague and by early 1957 Patočka had studied 37 additional cases in the same region In severe cases *Listeria* may be isolated without difficulty from blood cerebro spinal fluid urine and other secretions Potel (1951) has observed that the meconium in such cases is always positive perhaps due to swallowing of infected amniotic fluid These infants often die within 2 or 3 days after



Fig 44 Granulomatosis infantiseptica showing extensive miliary abscesses in the pharyngeal region (After Reiss H J Zur pathologischen Anatomie der kindlichen Listeriosis Kinderarztl Praxis 1953 2 12)

birth showing a generalized purpuric and petechial rash like that of meningococcal septicemia (Fig 45)

The pathogenesis of granulomatosis infanti septica is not clearly understood but frequently *Listeria* has been isolated postpartum and sometimes antepartum from the mother's vagina and occasionally from urine or blood Some of the mothers had noted at different times before delivery minor febrile illnesses on one or more occasions but none became seriously ill and most had a normal postpartum course Although the mortality is high in infants it is very low in the mothers who usually exhibit uncharacteristic or even inapparent infection which may be indicated only by a rising of agglutination titer It is suggested that infection in infants with this disease is of hematogenous origin through the relatively intact uterus of inapparently ill mothers Only 2 cases of granulomatosis infantiseptica have been reported to date in North America by Johnston et al (1955) from Toronto

Diagnosis

Human infections due to *L monocytogenes* have been reported with increasing frequency during the past decade This may indicate either an increased incidence of such infections or more *probably an increasing awareness* on the part of the physicians and the bacteriologists Murray (1955) states that 'a shrewd suspicion may be awakened by awareness of prevailing recognized cases, but the actual diagnosis depends upon the alertness of the bacteriologist Absolute dependence can only be placed on the isolation and identification of the organisms"

Cultures should be made from blood, cerebrospinal fluid, urine, meconium placenta lochia, milk or exudates, according to indications in the individual case and the available means of collecting specimens At autopsies cultures should be made from all organs since *Listeria* has been isolated from many tissues in the reported cases of granulomatosis infantiseptica Brain tissue, and particularly medulla oblongata should be treated by the technic of Gray et al (1948) Emulsified brain tissues are kept at 4° C for several weeks and subcultured from time to time All cultures should be made on blood agar and incubated at 37° C Care must be taken that colonies of *Listeria* are not confused with beta



Fig 45 Granulomatosis infantiseptica showing purpuric and petechial rash over entire body of infant (After Reiss H J Zur pathologischen Anatomie der kindlichen Listeriosis Kinderarztl Praxis 1953 2 12)

hemolytic streptococci and that they are not mistakenly discarded as diphtheroids particularly since they grow readily on media containing potassium tellurite Microscopy is essential and examination of hanging drops for characteristic tumbling motility must be performed In specific identification the reaction produced in the rabbit eye is of great value *Listeria* instilled into the conjunctival sac produces keratoconjunctivitis (Fig 46) (Julianelle 1941) The inoculation of experimental animals apart from the rabbit eye test is not too reliable since most laboratory animals are susceptible to the natural disease

The isolation of *Listeria* from some types of clinical specimens is very difficult due to overgrowth of other organisms particularly *Escherichia coli* Shimizu et al (1954) have overcome this difficulty to some extent by adding 1/10 000 guanofuracin to other plating medium This concentration permits growth of *Listeria* and inhibits to varying degrees the growth of contaminating bacteria Girard (1957) has used 1/5 000 furacin in tryptose phosphate broth for preliminary treatment of badly contaminated specimens followed by plating on phenylethanol blood agar This will enable detection of 10 to 100 *Listeria* per ml

Serologic methods may be used as an aid to the diagnosis of listeriosis in man but since the agglutination titer decreases rapidly after recovery it cannot serve reliably for late diagnosis One must also consider the fact that *Listeria* has common antigens with staphylococci and with *Streptococcus faecalis* (Seeliger and Sulzbacher 1956) Titers of 1/80 to 1/100 have been reported in approximately 20 per cent of sera from healthy children and adults (Krepler and Flamm 1956) There is no bacteriologic evidence that the majority of titers in normal healthy individuals as well as in domestic animals results from *Listeria* infections These facts must be considered when utilizing the agglutination test The test should be done with H and O antigens of the 4 serotypes of *L monocytogenes* and with the autogenous strain Titers of over 1/200 are significant but a rising titer on consecutive serum samples is even more significant



Fig 46 Listeria keratoconjunctivitis 9 days after instillation of *L monocytogenes* showing edema corneal opacity and purulent exudate (After Gray Singh and Thorp Jr Abortion stillbirth early death of young in rabbits by *Listeria monocytogenes* I Ocular instillation Proc Soc Exper Biol & Med *89* 163 169)

Specific Therapy

In vitro studies by Linzenmeier and Seeliger (1954) on a number of strains of *Listeria* indicate that the organism is sensitive as a rule to penicillin streptomycin chloramphenicol the tetracyclines and erythromycin but is resistant to sulfadiazine polymyxin and bacitracin These findings are borne out in varying degree in the actual treatment of clinical cases of listeriosis Sulfonamides have proved to be of little value Streptomycin is of limited value and apparently the organisms may develop resistance to this antibiotic rather rapidly Cases have been treated successfully with combined penicillin and streptomycin and with tetracycline Most cases of granulomatosis infantiseptica are fatal regardless of treatment and there have been so few reported cases of meningitis and septicemia that it is difficult if not impossible to assess claims which have been made for various methods of treatment In all cases the strain isolated should be tested in vitro for its sensitivity to antibiotics

Epidemiology

Listeriosis is a disease with a very widespread distribution involving many unrelated and varied hosts with widely different food

and living requirements, under every climatic condition from arctic to tropical The clinical pathologic and bacteriologic characteristics of listeriosis vary from one host to another but there is no indication that this is due to difference in species or in type of infecting bacteria The specific characters of strains of *Listeria* isolated from all hosts in every country appear to be homogeneous apart from the fact that the 4 different serotypes may be involved in different regions No serotype is restricted to any particular host in fact, strains isolated from cases not showing monocytosis as in cattle and generally in man, nonetheless cause a characteristic monocytosis in rabbits

Present information indicates that listeriosis in man at any rate is a disease with a low incidence and a high mortality rate This belief may be misleading because of the difficulty of isolating *Listeria* whether from the sick or the normal individual Because of its wide spread geographic distribution and its variety of hosts the organism in all probability has a high transmission rate and is able to persist for long periods of time in many individuals

It would appear that individual susceptibility or resistance might account for the situation since animal experiments have shown that susceptibility to listeriosis can be altered by environment and general state of health For example the weaning period of young rabbits is dangerous and outbreaks among domestic animals seem to be associated with fall and winter feeding but terminate when the animals are grazing outdoors Herd outbreaks in animals have been associated with introduction to the herd of animals from infected sources as might be expected Contamination of food is not effective but Gray et al (1955b) have demonstrated that the addition of *Listeria* to the drinking water of pregnant rabbits will result in abortion whereas non pregnant or male animals are completely refractory to this method of exposure These findings support the belief of Potel (1953) that the oral route is the avenue of entrance in the widespread outbreaks of granulomatosis infantiseptica in the human population studied in Germany He found that many of the mothers were taking cow or goat milk and in one instance he isolated *Listeria* from the milk of a cow with atypical mastitis A woman drinking milk from this cow gave birth prematurely to twins and *Listeria* was isolated from the liver of each Similar observations have been made in animal outbreaks

The oral route is apparently not the only portal of entry since Gray et al (1955a) have reported intra uterine infection of rabbits following ocular instillation of *Listeria* The venereal route must not be overlooked in the pathogenesis of listerial infection Wenkebach (1953) isolated *L monocytogenes* from the urethral exudate of 5 men with gonorrhea Although the micro organism appeared to have no pathogenic role in these instances the possibility of venereal transmission is suggested Respiratory exposure may also play a role particularly in encephalitis of ruminants and in septicemia and meningitis in man The organism has been isolated from the throat of sheep with listeric encephalitis and from a nasal swab of a normal sheep Stanley (1950) on examining 3 558 swabs from the human nose the throat or the nasopharynx isolated 2 cultures which showed morphologic cultural and biochemical properties similar to those of *L monocytogenes*

It has been suggested that inhalation and ingestion may be responsible for outbreaks of listeriosis in man and animals Some evidence had been presented to support these hypothesis in both instances but in man at any rate very little is known about the epidemiology of listeriosis Transmission may be from man to man or from animals to man While the brunt of the incidence in an outbreak may fall on the young in terms of mortality or morbidity nonetheless the infection or carrier rate in adults is entirely unknown and it is possible that the examination of the upper respiratory tract the gut and the genito urinary tract might provide interesting information in this connection

ERYSIPELOTHRIX RHUSIOPATHIAE

History

Erysipelothrix rhusiopathiae is the etiologic agent of erysipeloid a not uncommon cutaneous infection in man which on occasion may be confused with human erysipelas of streptococcal origin *Erysipelothrix* infections also occur commonly in swine and are referred to

as swine erysipelas A similar infection may occur in various fowl and is of some economic importance in turkey flocks The first member of this group was isolated by Koch in 1880 from the blood of mice and in 1886 by Loeffler from the cutaneous blood vessels of pigs that had died of swine erysipelas Rosenbach (1909) isolated *E rhusiopathiae* from human infections in 1886 and was the first to use the term erysipeloid for this disease in man While at one time strains of human porcine and murine origin were considered separately it is now generally held that these are almost identical variants of the single species *E rhusiopathiae*

There are some similarities between *E rhusiopathiae* and *L monocytogenes* which have led Topley and Wilson (1956) to place both organisms in the genus *Erysipelothrix* However this proposal is not widely accepted in North America since there are differences in both motility and pathogenicity between the two organisms and since there appears to be no antigenic relationship between the two Woodbine (1950) has reviewed the bacteriology and the chemotherapy of *Erysipelothrix*

Morphology and Cultivation

E rhusiopathiae is a gram positive nonsporulating rod in which no capsule has been demonstrated The organism is micro-aerophilic but will grow either aerobically or anaerobically Like *Listeria* it occurs in a smooth and a rough form but differs from *Listeria* in being nonmotile Smooth phase cells are small straight or slightly curved rods with rounded ends measuring 0 8 to 2 5 microns in length and 0 3 micron in width and arranged singly in small clusters or in short chains In the rough form long filaments of up to 60 microns or more in length predominate and long chains of bacilli may also be seen

E rhusiopathiae grows poorly on simple media in contrast with *Listeria* but growth is improved by the addition of glucose and serum Smooth forms grow better at 33° C while at 37 C growth of the rough variant is favored After 24 hours incubation at 37 C smooth phase colonies are round glistening water clear and up to 0 1 mm in diameter On further incubation *Erysipelothrix* colonies show little or no increase in size while those of *Listeria* become larger In the rough form the colonies are larger and flatter with a matt surface and a fimbriate edge which make them not unlike minute anthrax colonies The formation of a lateral outgrowth in gelatin stab cultures gives rise to the characteristic lamp-brush appearance which when present is a diagnostic feature although it is not a consistent finding Gelatin is not liquefied Most strains of *Erysipelothrix* produce partial or alpha hemolysis around deep colonies in blood agar although a soluble hemolysin is not formed (Barber 1939)

Erysipelothrix is considerably less active biochemically than *Listeria* It usually forms acid from glucose lactose and sometimes mannose It does not ferment maltose rhamnose mannitol sucrose dextrin or salicin Indole methyl red Voges Proskauer and H O tests are negative (Barber 1939 Julianelle 1941)

Antigenic Structure

There is general agreement that *Erysipelothrix* and *Listeria* are antigenically distinct Watts (1940) recognized 2 distinct antigenic types of *Erysipelothrix* He found that each group possessed a heat stable specific antigen and apparently 2 heat labile antigens which were present in different proportions in the 2 groups and were responsible for cross agglutination Gledhill (1945) found evidence of heat stable somatic antigens which differed qualitatively and of a heat labile somatic antigen resistant to ethanol On the basis of the heat stable antigens it was possible to distinguish at least 4 different antigenic types The same series of antigens are apparently possessed by strains of both human and animal origin

Resistance

Exposure to moist heat for 15 minutes at 55° C will kill most strains of *Erysipelothrix* (Barber 1939) The organism is resistant to salting pickling and smoking surviving in such treated meat for 1 to 3 months It may also remain viable and virulent for months in putrifying infected carcasses although it is very susceptible to drying The organism will survive for 4 or 5 days in drinking water and for 12 to 14 days in sewage

and living requirements, under every climatic condition from arctic to tropical The clinical, pathologic and bacteriologic characteristics of listeriosis vary from one host to another but there is no indication that this is due to difference in species or in type of infecting bacteria The specific characters of strains of *Listeria* isolated from all hosts in every country appear to be homogeneous apart from the fact that the 4 different serotypes may be involved in different regions No serotype is restricted to any particular host, in fact, strains isolated from cases not showing monocytosis as in cattle and generally in man nonetheless cause a characteristic monocytosis in rabbits

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into the conjunctival sac of a rabbit *Listeria* gives rise to a severe keratoconjunctivitis whereas *Erysipelothrix* causes a very mild conjunctivitis without keratitis (Julianelle 1941). In suspected cases of septicemia repeated blood cultures should be done. No information is available regarding the positive diagnostic value of agglutination tests in man although in swine such tests have been used in diagnosis of the chronic form of infection.

Treatment

The evaluation of therapy in *Erysipelothrix* infections is complicated by the fact that the disease is usually self limiting and runs a variable course with an average duration of about 3 weeks. Both experimental and clinical studies have demonstrated that penicillin is the antibiotic of choice (Woodbine 1950). Streptomycin and chlortetracycline are less effective (Klauder 1953). Moynihan and Stovell (1954) on the basis of in vitro and in vivo tests found that in turkeys experimentally infected with *E. rhusiopathiae* penicillin oxytetracycline and a combination of penicillin and streptomycin were therapeutically effective. Streptomycin alone and sulfonamides alone had little therapeutic effect. Klauder et al. (1943) believe that in human cases with septicemia administration of immune serum should be considered.

Epidemiology

Erysipeloid in man is primarily an occupational disease seen in individuals handling meat fish poultry or shellfish. King (1946) summarized 115 cases treated at a London hospital and found that 85 of these were directly attributed to an animal source. Abattoir workers and fish handlers predominated in a group of 100 cases studied by Klauder (1938). Outbreaks of the disease have been described in workers in a bone button factory and one veterinary student became infected while dissecting a horse. However erysipeloid also has been observed as a nonoccupational disease in sporadic cases.

Although in man inoculation almost invariably takes place through broken skin in swine other portals of entry apparently are possible. Rowsell in experimental swine erysipelas found that various routes of experimental infection produced tonsillar carriers in 92 per cent of the pigs. He believes that natural infection in swine probably occurs by the oral route in which case danger to the herd of the tonsillar carrier of *Erysipelothrix* is obvious.

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Distribution and Range of Pathogenicity

E rhusiopathiae has a wide range of pathogenicity for animals under natural conditions and has a world wide distribution Natural infections have been reported in man, swine sheep mice, cattle, horses and various species of domestic fowl The organism may be found in decomposing nitrogenous material and remains viable and virulent for many months Mice and pigeons are highly susceptible to experimental infection while guinea pigs are not although the latter are susceptible to infection with *Listeria*

Pathogenesis and Symptomatology

While *Erysipelothrix* like *Listeria*, may produce a generalized septicemia with focal necrosis of liver and spleen in susceptible animals it differs from *Listeria* in manifesting a predilection for the skin the endocardium and joints

Infection in swine is commonly seen in 3 different forms a mild form known as red fever, diamond skin disease or ' swine erysipelas in which skin involvement predominates an acute severe form of septicemia and a chronic form characterized by arthritic symptoms *Erysipelothrix* infections of swine are often serious and fatal and may have considerable economic importance Infection of commercial turkey or chicken flocks is characterized primarily by involvement of the long bones from which positive cultures may be obtained The mortality rate in such flocks may be reduced materially in a potential epidemic threat by the use of a vaccine

The disease in man usually takes the form of a localized cutaneous infection known as Rosenbach s erysipeloid, although rarely septicemia with endocarditis and joint involvement may occur (Klauder et al 1943) Interest in erysipeloid in North America dates from a report of Gilchrist (1904) in which 329 cases were seen in Baltimore 232 were individuals who had suffered abrasions while handling crabs The infection characteristically develops following an abrasion suffered while handling organic matter, especially fish shellfish meat or poultry (Klauder 1938) Erysipeloid commonly appears as a mild cutaneous infection 1 to 7 days after inoculation usually in a finger or a thumb A small red sharply defined and slightly elevated spot appears at the site of inoculation and gradually spreads outward, while the central area fades and takes on a purplish hue This is accompanied by edema and erythema and is followed by throbbing or tingling in the involved area A mild headache and malaise may be present. Suppuration does not occur, and lymphangitis and lymphadenitis are irregular In about 6 per cent of patients arthritis may involve the injured area or adjacent joints Very rarely, infection may become systemic and develop into an *Erysipelothrix* septicemia which can be fatal In such an event there is seen a generalized purpuric and petechial rash resembling the rash of meningococcal septicemia A single attack does not confer complete immunity in man and reports of reinfections are common

Spencer (1954), during studies with experimental swine erysipelas, found that the skin was the preferred site for a primary invasion of *Erysipelothrix* and concluded that the disease was more likely to develop in swine whose resistance was lowered by concurrent infection Rowsell (1955) tried to produce symptoms of natural infection in swine by scarification of the skin intravenous injections and oral administration and found only the latter method to be effective Under the conditions of his experiments animals could not be reinfected once they had recovered

Diagnosis

A specific diagnosis of erysipeloid is based in part on visual clinical findings Characteristic appearance of the localized infection which almost invariably is on the hand and the absence of suppuration leukocytosis and systemic involvement, all aid in differentiating human erysipeloid from cellulitis and streptococcal erysipelas However final diagnosis of erysipeloid rests on the isolation of *E rhusiopathiae* from lesions Positive cultures are rarely obtained from material collected from swabs of a local lesion It is recommended by Barber et al (1946) that a biopsy be taken and cultured for 24 hours in glucose broth followed by subculture on blood agar plates Intraperitoneal injection of white mice with a suspension of biopsy material will yield a pure culture of *Erysipelothrix* from heart s blood in 24 hours The organisms can be differentiated from *Listeria* by biochemical reactions and by the fact that on instillation

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21

The Cholera Vibrios

The *vibrios* are curved rods possessing a single polar flagellum They are highly motile non spore forming gram negative and facultatively aerobic

V ibrio cholerae is the specific cause of the disease cholera in man and does not infect other hosts in nature It is nonhemolytic usually ferments sucrose and mannose but not arabinose grows at higher pH than other enteric pathogens and possesses a specific group somatic antigen (Synonym *V comma*)

HISTORY

Long before bacteria were discovered the features of cholera epidemics had been explored thoroughly and the existence of minute living causative organi ms was postulated In 1854 Snow through masterly detective work in the Broad Street Pump epidemic in London incriminated a contaminated water source This is a landmark in epidemiologic research The well known Hamburg Altona epidemic of 1892 indisputably established the theory of water borne disease The cholera vibrio was discovered by Koch in 1883 and many of the basic phenomena of bacteriology and immunology were first een and described in studies of that organism during the next few years In 1893 Pfeiffer observed that guinea pigs which had been immunized with *V cholerae* were able to destroy the organism on reinjection (immune bacteriolysis) and he discovered that this power could be conferred upon normal guinea pigs by injecting into them serum from immunized animals Bordet shortly thereafter produced the Pfeiffer phenomenon in vitro and demonstrated that a heat labile substance complement or alexine present in normal serum was essential to the reaction The precipitin reaction was first described (1897) by Kraus who added broth filtrates of cholera cultures to specific immune sera

MORPHOLOGY AND STAINING

Freshly isolated vibrios are comma shaped about 0 3 μ broad and 4 μ long (Fig 2M) they possess a single polar flagellum and are highly motile Occasionally 2 or 3 vibrios are attached end to end and assume the form of a spirillum When grown on artificial media for prolonged periods they may lose their characteristic comma shape and become straight rods indistinguishable morphologically from other enteric bacilli They are gram negative and can be stained by the usual aniline dyes The flagellum is seen only by means of special staining procedures or with the electron microscope

GROWTH REQUIREMENTS AND CULTIVATION

Vibrios including those causing cholera can be grown on a medium containing a few mineral salts with asparagin as the sole source of carbon and nitrogen They grow well on all the usual laboratory media and prefer a temperature of 37 C Most abundant growth is obtained in the presence of gaseous oxygen but some strains will grow anaerobically They will grow at a high pH (9 0 to 9 6) but are

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cell bodies can be used to produce pure anti H sera Unlike the somatic antigen the H antigen is not modified in dissociation from the smooth to the rough form and its antibodies do not appear to be involved in the protection of susceptible animals

THE SOMATIC ANTIGENS

Gardner and Venkatraman (1935) found that the vibrios can be divided into 6 major subgroups on the basis of their somatic antigens Almost all the vibrios from typical cases of cholera belong to subgroup I Vibrios of subgroups II to VI were isolated from water or from atypical cases of diarrhea This classification has been very useful and has been adopted universally

The subdivision of cholera vibrios (O subgroup I) into serologic types is a subject on which opinions differ Early Japanese workers described 3 variants (Inaba Ogawa and Hikojima) which they identified by agglutination reactions using absorbed antisera Inaba possessed one type antigen (A) Ogawa another (B) and Hikojima possessed both (AB) Burrows et al (1946) supported by Gallut have indicated that there may be many additional somatic antigens but this is disputed by Kauffmann (1950)

The soluble somatic antigens extracted from smooth cholera vibrios are complex molecules of the complete antigen type They are antigenic toxic and possess polysaccharide fractions which confer upon them serologic specificity The union between the somatic antigens (as they exist in the intact cell) and the corresponding antibodies results in agglutination reactions of the rapid finely granular type and bacteriolysis occurs in the presence of complement When properly extracted from the bacteria these antigens give well defined precipitin tests Antibodies for the somatic antigens also protect laboratory animals from otherwise lethal injections of vibrios In the case of chick embryos protection is conferred both by group and type antibodies and the same is doubtless true in other hosts

The first immunochemical work on the vibrios was done by Landsteiner and Levine (1917) They isolated an alcohol soluble carbohydrate haptene which reacted in a precipitin test White (1936) investigated the immunochemistry of the somatic antigens and identified 4 polysaccharide haptenes which he named Cα Cβ Cγ and Cδ In the normal smooth state all 4 polysaccharides are present and Cα is dominant in agglutination reactions In the rough state Cα is lacking and Cβ is dominant In the ρ state only Cγ and Cδ are present and Cδ is dominant Serologically specific differences in Cα are responsible for the Japanese types Cα (Inaba) and Cα (Ogawa) Linton (1940) criticized White s work believing that some of the above polysaccharide fractions were chemical artifacts He agreed with White that the change from smooth to rough was characterized by a loss of the S polysaccharide but maintained that the rough strains possessed a new polysaccharide which was not represented even *in a concealed form in smooth strains*

White (1940) described a number of additional antigens of the cholera vibrios (1) A heat labile protein antigen detectable by a precipitin reaction but not participating in agglutination reactions which was found in all vibrios but not in other organisms (2) a heat stable protein antigen likewise participating in precipitin but not in agglutinin reactions and present in vibrios of various dissociative states (3) an alcohol soluble Q protein similar to that found in the Salmonellas and (4) a rugose haptene the intercellular substance of rugose variants which reacted in agglutination and precipitin reactions

Linton (1940) approached the classification of the vibrios from a novel point of view He isolated 2 proteins differing in optical rotation and 3 polysaccharides differing in the hexose obtained on hydrolysis (glucose galactose or arabinose) By grouping each of the proteins with each of the polysaccharides he divided the vibrios into 6 groups which should not be confused with the 6 serologic groups of Gardner and Venkatraman Linton s classification has not been *widely used and the significance of his groups* in relation to the recognized serologic attributes of various vibrios is not known

TOXINS

Vibrios produce an endotoxin which shares properties with the endotoxins of other gram negative organisms (see Thomas 1954) Toxic extracts have been made of cholera vibrios that are lethal for mice rabbits and guinea pigs but protective antisera have not been developed Burrows et al (1944) have reported extraction of a toxin that increases

very sensitive to acid In the presence of fermentable carbohydrate a culture of vibrios will quickly sterilize itself, due to acid production

The colonies of smooth organisms grown on meat infusion peptone agar are semitranslucent or opaque moist and with an entire edge The surface may be smooth or finely granular Rough colonies may show an irregular edge and a more granular surface but often they are indistinguishable from smooth colonies The colonies of the variant known as rugose possess radical or irregular corrugations of a very striking sort correlated with the secretion by the culture of a gelatinous intercellular substance The rugose form is entirely unrelated to S → R variation

BIOCHEMICAL ACTIVITIES

Although no precise classification of vibrios on the basis of their fermentations has been achieved the regularity with which strains from cholera ferment mannose and sucrose *but fail to ferment* arabinose (*Heiberg 1935*) justifies the determination of these biochemical activities in the identification of the cholera vibrios Cholera vibrios produce indole from tryptophane and reduce nitrate When concentrated sulfuric acid is added to a culture in which these reactions have occurred a red pigment is formed This is the cholera red test A positive reaction is given by almost all cholera vibrios but is not limited exclusively to them and hence has little diagnostic significance In performing the test a peptone must be used which contains adequate tryptophane and nitrate such as Difco Tryptone The presence of glucose in the medium inhibits the reaction

Some of the vibrios produce a soluble hemolysin which can be detected by adding 24 hour broth culture to a 3 per cent suspension of washed goat erythrocytes The reaction is read for hemolysis after 2 hours incubation at 37 C and after standing overnight in the icebox Sometimes strains which do not form the soluble hemolysin produce a slow incomplete hemolysis on blood agar plates This delayed hemolytic action has been attributed to a proteolytic enzyme *V cholerae* does not produce the soluble hemolysin but may cause the delayed type of hemolysis

RESISTANCE

Cholera vibrios are highly sensitive to acid and it is doubtful if they can live more than a few moments in gastric juice which contains free hydrochloric acid They are killed by exposure to 56° C for 15 minutes, and they succumb to the usual chemical disinfectants They are very sensitive to drying but are not adversely affected by diffuse daylight

CHOLERAPHAGE

Many races of bacteriophage active against cholera vibrios have been recovered from water, sewage or the stools of patients These choleraphages have been used extensively in therapy and in the treatment of sewage and water, with equivocal results It has been suggested that choleraphage plays an important role in the recovery of patients from the natural disease When bacteriophage is added to a culture of vibrios in due time resistant variants appear which may differ from the original strain in colony form antigenic structure dissociative state agglutinability and hemolysin production The action of a race of phage is directed toward some particular constituent of the bacterial cell and is specifically inhibited by adding an excess of that constituent to the medium

ANTIGENIC STRUCTURE AND DISSOCIATION

The vibrios undergo dissociative changes of the S → R type and in addition show a further degradation to the ρ form Alterations in colony form are not necessarily conspicuous in this dissociation, but a modification of antigenic structure occurs which is *discussed* below The rough variants agglutinate spontaneously in 0 85 per cent saline solutions and are agglutinated by acriflavine

THE FLAGELLAR ANTIGEN

The vibrios possess a single flagellar antigen (the H antigen) which is identical serologically in all the true cholera vibrios and in many of the noncholera vibrios The flagellar H antigen is heat labile, being destroyed by exposure at 100 C for 2 hours Immune sera prepared with heated vaccines contain antibodies for the somatic antigens but not for the H antigen Suspensions of flagella which have been separated mechanically from the

the specific diagnosis must be made bacteriologically

In patients who recover bactericidal antibodies and agglutinins appear in the blood Elements which agglutinate very dilute suspensions of vibrios appear in the fecal contents of animals injected parenterally or infected per os experimentally with the vibrio (Burrows et al 1948) These elements have been termed fecal or coproantibodies but neither their exact nature nor their possible role in protection against natural infection has yet been made clear Their early appearance after infection or injection and their failure to vary in concentration with serum agglutinins differentiates them from the latter

ACTIVE IMMUNITY

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The vibrios will grow on any of the media usually employed for the isolation of enteric pathogens but where cholera is suspected clinically it is advantageous to use a medium which preferentially encourages vibrionic growth Such media depend in the main on the ability of vibrios to grow at a pH so high that other organisms likely to be encountered in stools are inhibited A modified Wilson and Riley medium has been most successful in India Whenever possible stool should be plated directly on this medium but when a considerable period of time must elapse between collection of specimens and cultivation a buffered saline preserving medium should be employed In the case of contacts and convalescents when the number of vibrios in the specimens may be relatively small a preliminary enrichment in Read s modification of Wilson and Blair s broth is desirable

During an active epidemic colonies suspected of being V *cholerae* may be identified tentatively by testing the organisms from the colony for agglutination on a slide with a suitable diagnostic serum Confirmation is obtained by macroscopic agglutination In sporadic cases particularly in areas where cholera is not usually prevalent identification of the strain should include study of hemolysis fermentation reaction and reaction in the cholera red test as well as serologic characteristics Details for isolation and identification of V *cholerae* are given in W H O Technical Rep Ser No 18 1950

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TREATMENT

The treatment of cholera is directed toward the patient s disturbed physiology rather than toward the vibrio and the parenteral admin

the rate of flow across whole rabbit and guinea pig intestine used as a membrane

It has been shown by Burnet and Stone (1947) that filtrates of *V cholerae* contain enzymes which in vitro cause desquamation of the intestinal mucosa of guinea pigs One of these enzymes has been demonstrated to be a mucinase Together they may play a part in the pathogenesis of cholera Burnet McCrea and Stone (1946) have also demonstrated an enzyme in cholera filtrates which destroys the receptors of red blood cells for virus particles It is distinct from the desquamative enzymes and has no apparent pathologic role

THE EL TOR VIBRIOS

A variety of vibrio first isolated from pilgrims in 1905 at the Tor quarantine station is known to differ from the true cholera vibrios only in that it produces a soluble hemolysin It is apparently capable of producing epidemics of diarrheal disease

DISTRIBUTION AND RANGE OF PATHOGENICITY

The cholera vibrios infect only man in nature Rabbits guinea pigs and mice can be killed experimentally by large parenteral inoculations Koch produced infections in rabbits by feeding the inoculum after paralyzing intestinal motility with opium and neutralizing gastric acidity Freter has shown that if guinea pigs are starved several days given $CaCO_3$ by mouth and 3 hours later a culture of streptomycin resistant *V cholerae* plus streptomycin plus $NaHCO_3$ by mouth an infection is produced that resembles the human infection in several ways It is localized in the intestines heart and spleen cultures remaining negative it is often fatal and large amounts of fluid pour into the lumen of the bowel Apparently the important factor in the procedure is suppression of normal gut micro organisms by streptomycin allowing the vibrios to become established Developing chick embryos are highly susceptible to small inocula of vibrios but the infection produced has little relation to human cholera

The survival of cholera vibrios outside of the host is favored by dampness and low temperatures but in any case it is not of long duration The vibrios remain viable in rice water stool and in river water up to 16 or 17 days, and in sea water as long as 4 days They have been recovered from septic tank contents up to 5 days and from a few hours to 2 weeks after experimental contamination of food These periods are long enough to allow transfer of infection but cannot account for the persistence of organisms through interepidemic periods

A natural disease of chickens is caused by *Vibrio metchnikovi* which closely resembles the cholera vibrio but is antigenically distinct from it Vibriolike organisms have been recovered from exudates of aborting sheep and cattle It should be mentioned that hog cholera and chicken cholera are not caused by vibrios

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Cholera is acquired by the ingestion of cholera vibrios from contaminated water or food or from direct contact with a cholera victim The ingested vibrios reach the intestine where bacterial multiplication occurs rapidly The gut wall is irritated and responds with a profuse outpouring of fluid This is accompanied by diarrhea and leads to profound dehydration of the patient The stools lose their normal fecal character and resemble rice water The vibrios remain localized in the intestinal tract and blood stream invasion is unknown Whether there is a systemic absorption of cholera toxin is a matter of dispute The clinical picture of cholera is one of dehydration hemoconcentration toxemia and finally shock Death may occur a few hours after the onset of symptoms In the most rapidly fatal cases there is insufficient time for extensive pathologic change to take place and little may be seen at autopsy except the rice water stools edema of the gut wall and dehydration of the tissues In more prolonged cases the mucosa of the small intestine is deeply injected and may show areas of sloughing Sometimes most of the small bowel is denuded of its epithelial lining The kidneys may show a toxic nephrosis Microscopically the small intestine shows hyperemia and sometimes loss of epithelium but there is little cellular reaction to the infection Vibrios are present abundantly on the mucosal or the denuded surfaces of the gut but they do not penetrate the submucosa There are no distinctive pathologic findings in cholera and

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istration of fluids constitutes the most important measure V. *cholerae* is sensitive in vitro to the various sulfonamides, streptomycin chloramphenicol chlortetracycline and oxytetracycline but adequate clinical studies have not been done to establish their positions in therapy and prophylaxis There are no specific antisera or antitoxins used in the treatment of cholera

EPIDEMIOLOGY

Cholera has been endemic in India for many years In 1817 it started on the first of a series of epidemic waves which continued irregularly throughout the 19th century Each pandemic extended further from the endemic source until Europe was reached (1830) and finally America (1832) There has been no cholera in America since 1892 or in Europe since 1925 In 1947 Egypt was reached Epidemics continue to appear with some regularity in Asia In India there were 337,000 deaths from cholera in 1930 More than 200 000 cases were reported in India and Pakistan in 1950 Recently small epidemics have occurred in China and the disease seems to have become endemic in Burma Thailand and French Indo China Calcutta is the most heavily and persistently infected port city in the East The world wide dissemination of cholera in the early epidemics closely followed shipping lanes overland trade routes and pilgrim migrations The disease was carried from place to place by individuals who had mild cases of the disease who were incubating it or were carriers Cholera is spread by contaminated water food flies and by direct contact The explosive water borne outbreaks in Hamburg and England have overemphasized the role of water in the spread of cholera In India water is by no means the inevitable source of dissemination The Egyptian epidemic of 1947 appears to have originated from contaminated dates

Local variations in the manifestations of cholera are striking Case fatality rates in the Madras presidency for example are considerably higher than in Bengal although incidence rates in the population at large are generally lower than in Bengal

The carrier state in cholera is usually of short duration and chronic carriers such as occur in typhoid fever are unknown The convalescent carrier is usually free of vibrios in a few days, almost invariably in a month In water, the organisms may last 2 weeks Therefore the survival of cholera vibrios through interepidemic periods is not easy to explain but it probably depends on sporadic cases in the endemic areas

The character of the epidemic does not vary significantly with the serologic type of cholera vibrio present In some epidemics a single type may be found, in others there may be a mixture of types In India Ogawa epidemics are common, in Japan there have been pure Inaba epidemics and in China Hikojima has predominated However there are no rigid geographic associations and in a given locality the type may vary from epidemic to epidemic A recent epidemic in Celebes was caused by El Tor vibrios and it carried the high mortality associated with true cholera

CONTROL MEASURES

Cholera is a disease which occurs only under the most deplorable sanitary conditions Filtration or treatment of water proper disposal of excreta and sanitary handling of food makes it impossible for cholera to spread in a community

Where, for various reasons adequate sanitation cannot be achieved, mass vaccination has been tried Unfortunately, it is still impossible to judge its value precisely although cholera vaccine has been used for more than 50 years The largest experiments were conducted in the Balkans during World War I and in India in 1942 1944 (Adiseshan Pandit and Venkatraman, 1947) In the Indian epidemic 1 180 000 out of a population of 3 000 000 were given a single inoculation during the epidemic The attack rates were considerably lower in the inoculated group but case mortality rates were not affected favorably In this epidemic as in previous ones the observations were made under difficult circumstances and the inoculated and the uninoculated groups were not strictly comparable Further experience under properly controlled conditions is necessary to settle the question The possibilities of chemoprophylaxis in cholera have not been explored adequately

Rapid air travel increases the possibility

of introducing cholera into North America and Western Europe from the Orient but it is unlikely that it would become established *or spread in these areas The American Pub*lic Health Association and the British Ministry of Health recommend as control measures the isolation of patients disinfection of excreta quarantine of contacts for 5 days from last exposure immunization of contacts bacteriologic detection and isolation of carriers and rigid sanitary measures with respect to water and food and fly suppression

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Local variations in the manifestations of cholera are striking Case fatality rates in the Madras presidency for example are considerably higher than in Bengal although incidence rates in the population at large are generally lower than in Bengal

The carrier state in cholera is usually of short duration and chronic carriers such as occur in typhoid fever are unknown The convalescent carrier is usually free of vibrios in a few days, almost invariably in a month. In water, the organisms may last 2 weeks. Therefore, the survival of cholera vibrios through interepidemic periods is not easy to explain, but it probably depends on sporadic cases in the endemic areas

The character of the epidemic does not vary significantly with the serologic type of cholera vibrio present In some epidemics a single type may be found in others there may be a mixture of types In India Ogawa epidemics are common in Japan there have been pure Inaba epidemics, and in China Hikojima has predominated However there are no rigid geographic associations, and in a given locality the type may vary from epidemic to epidemic A recent epidemic in Celebes was caused by El Tor vibrios and it carried the high mortality associated with true cholera

CONTROL MEASURES

Cholera is a disease which occurs only under the most deplorable sanitary conditions Filtration or treatment of water, proper disposal of excreta and sanitary handling of food makes it impossible for cholera to spread in a community

Where for various reasons adequate sanitation cannot be achieved, mass vaccination has been tried Unfortunately it is still impossible to judge its value precisely, although cholera vaccine has been used for more than 50 years The largest experiments were conducted in the Balkans during World War I and in India in 1942 1944 (Adiseshan Pandit and Venkatraman 1947) In the Indian epidemic 1 180 000 out of a population of 3 000 000 were given a single inoculation during the epidemic The attack rates were considerably lower in the inoculated group but case mortality rates were not affected favorably In this epidemic, as in previous ones the observations were made under difficult circumstances and the inoculated and the uninoculated groups were not strictly comparable Further experience under properly controlled conditions is necessary to settle the question The possibilities of chemoprophylaxis in cholera have not been explored adequately

Rapid air travel increases the possibility

distinguishing the true influenza bacillus from closely allied species

However certain facts were learned from the extensive bacteriologic investigations carried out during the pandemic of 1918 Davis (1917 1924) Thjotta and Avery (1921) Fildes (1921) and Rivers and Poole (1921) extended our knowledge of bacterial growth factors and standardized procedures for the use of X and V factor requirements as a diagnostic aid In brief they showed that whole blood contained both factors Their action could be separated by exposing whole blood extracts to 250° F X factor was thus destroyed A yeast extract sterilized by filtration served as a good source of V factor

A study of the nutritional requirements of strains diagnosed as influenza bacilli during the pandemic of 1918 led to the discovery of some new organisms Pritchett and Stillman (1919) reported an organism which they labeled X bacillus a beta type of hemolysis appeared following the growth of this organism on blood agar and X factor was not needed for growth Rivers (1922) described strains which he named *B parainfluenzae* they differed from true *H influenzae* only in their ability to grow in the absence of X factor Hemolytic strains requiring both X and V factors have been reported by both Fildes (1924) and Valentine and Rivers (1927)

Search for evidence of a filtrable virus in patients with influenza also met with failure as outlined by Jordan (1927) and Scott (1929) Technics for the isolation and the identification of viruses were just beginning to be explored It is believed that the choice of patients too late in their disease to yield the virus and the use of immune individuals as recipients were responsible for the failure to transmit the virus to human beings Nonetheless the view was held by a number of investigators of the 1918 pandemic that its unprecedented severity reflected the concurrent interplay of a virus and the influenza bacillus This thesis was strengthened by the recovery of a virus from swine influenza (Shope 1931) and by the demonstration that the synergistic effect of an influenza bacillus *H suis* and swine influenza virus is essential for both the natural and the experimental disease The importance of this contribution deserves emphasis it illustrates the enhancement of injury caused by the combined effect of a bacterial and a virus infection

The investigations of Shope (1931 1944) raise the question whether the facts disclosed for swine influenza also hold true for human pandemic influenza Since the swine epidemics appeared for the first time concurrently with the human influenza pandemic of 1918 Shope suggests that the latter disease originated in swine A study of the epidemiology of the swine disease demonstrated that the virus lies dormant in lung worms which appear to live in symbiotic relationship in the swine lungs Under appropriate climatic conditions *H suis* is found in increasing numbers in the nasopharynx of the experimental animals The virus ceases to lie dormant and epidemics of swine influenza are launched

The clinical and pathologic similarities of swine and human influenza have led several investigators to explore the synergistic action of human influenza viruses and *H influenzae* the conflicting results have been reviewed and extended by Bang (1943)

The effect of *H influenzae* on both human and swine virus deserves re examination in light of current concepts concerning the biology of *H influenzae* However it is evident from our present knowledge that the human and the swine influenza viruses as well as the two varieties of *Hemophilus influenzae* and *suis* possess fundamental differences Even if it could be shown that the swine virus had been the primary cause of the 1918 human pandemic subsequent epidemics of influenza have clearly been the results of different viruses Evaluation of the importance of synergism between an influenza virus and a bacillus in determining the severity of human pandemics must await additional study

Role of *H Influenzae* as a Primary Pyogenic Agent

The first authentic case of influenzal meningitis was reported by Slawyk (1899) The importance of this organism as a cause of meningitis was emphasized by Rivers (1922) Lemierre (1936) first described the characteristic clinical syndrome which follows when *H influenzae* produces an obstructive infection of the lower respiratory tract

Some fundamental differences between strains cultivated from these pyogenic infec

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22

The Hemophilus Group

HEMOPHILUS INFLUENZAE

Hemophilus influenzae the type species of the Hemophilus group has played two important roles in human infections (1) a secondary role in pandemic virus influenza and (2) a primary role producing pyogenic infections the latter occur infrequently in adults in children on the other hand *H influenzae* is one of the more frequent causes of serious pyogenic infections

The members of the Hemophilus group are gram negative nonmotile nonsporebearing aerobic bacilli and lead a strictly parasitic existence they possess poorly developed enzyme systems therefore all of them require enriched media Their nutritional needs yet imperfectly defined display certain differences within the genus which have a limited but useful application in identification The hemophilic bacteria have been classified as in Table 46 on the basis of their needs for two growth factors X and V and other biologic traits

Role of *H Influenzae* in Pandemic Influenza

During the pandemic of influenza of 1890 Pfeiffer (1892, 1893) isolated from the nasopharynx of most of those suffering from the disease small straight gram negative bacilli tending to occur in clumps they stained with difficulty by ordinary dyes Loeffler s methylene blue revealed polar granules Growth in pure culture required substances present in whole blood the growth stimulating substances were associated with the iron containing portion of hemoglobin Blood agar plates sparsely seeded yielded small transparent colonies which produced no change in the surrounding medium

The frequency of occurrence of this organism in patients with influenza and its alleged virtual absence in normal individuals led to the erroneous conclusion that *H influenzae* was the cause of the influenza pandemic of 1890 thus this organism was named the influenza bacillus and in 1923 was designated *H influenzae* by The American Society of Bacteriologists Those who have objected to this terminology have continued to use the name Pfeiffer s bacillus

Investigations on the role of this organism in epidemics from 1892 to 1920 are reviewed by Khristensen (1922), Scott (1929) and Jordan (1927) The results cast doubt upon the primary agency of the influenza bacillus in pandemic influenza

During the 1918 influenza pandemic extensive bacteriologic investigations were carried out to determine the role of the influenza bacillus The results are reviewed by Jordan (1927) and Scott (1929) There is no doubt that most investigators who studied this special problem and therefore had special interest in looking for *H influenzae* found a very high incidence not only in the nasopharynx but also in postmortem lung cultures Unfortunately, the methods then available could not differentiate between the encapsulated and thus potentially pathogenic influenza bacilli and the nonencapsulated forms known to be widely distributed in the normal nasopharynx Nor had methods then been developed for

sponsible For these reasons the vaccine used to produce rabbit antibody was prepared from the luxuriant growth of *H influen ae* resulting from 6 hours incubation of culture on Levinthal agar The organisms if washed from the plate with 0 5 per cent formalinized saline and immediately iced exhibited good preservation of capsules for as long as a few weeks However degeneration of capsules occurred even in the presence of 0 5 per cent formalin if the suspensions were allowed to stand at room temperature for as short a period as 2 hours

Heidelberger s quantitative chemical method was used for measuring antibody to *H influen ae* in rabbit antiserum the antibody concentration could be expressed accurately in terms of mg of antibody nitrogen per cc (Alexander and Heidelberger 1940) The use of selective dosage of antibody according to the severity of the meningeal infection permitted a quantitative approach to serum therapy

The development of a satisfactory virulence test for *H influen ae* by Fothergill et al (1937) provided an in vivo method for evaluating the influence of some therapeutic agents on this organi m In our experience (Alexander and Leidy 1943a) 2 to 200 organisms of type *b H influen ae* strains isolated from patients with *H influen ae* infections when suspended in mucin as first described by Miller (1933) are lethal for at least 50 per cent of mice infected by the intraperitoneal route Mouse protection tests were used to check the validity of the quantitative chemical methods for determining potency of type *b* rabbit antiserum by measuring the anticarbohydrate antibody The protective element in the therapeutic antiserum proved to be the anticarbohydrate (Alexander et al 1944) The power of available agents to protect against the experimental mouse infection has also served a a good guide to their therapeutic efficacy in human disease The results will be discussed later under Treatment

Morphology

Any description of morphology must be related to the source of the organisms In pathologic fluid spinal (Fig 47) synovial or pleural the organisms are usually predominantly coccobacillary simulating diplo

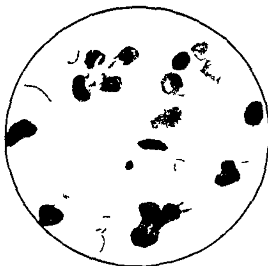


Fig 47 Gram stain of purulent spinal fluid infected with *H influen ae* type b × 800

cocci an erroneous diagnosis of pneumococcus is often made when Gram staining is unsatisfactory At times the bacilli occur in short chains and are so short that they are mistaken for streptococci Along with these forms it is virtually always possible to find definite bacilli some quite long at one end of some there is seen a spherical body stained only at the periphery Occasionally the predominant shapes are very bizarre long slender forms occurring together with thick bacilli which assume the contour of a club an elbow or other irregular outlines The possibility that these are variants which favor the emergence of a rough strain is suggested but demonstration of their capsules makes such an interpretation unlikely

In cultures the composition of the medium and the age of the culture determines to a great extent the morphology of *H influenzae* When Levinthal agar is seeded with 0 5 cc of Levinthal broth culture and incubated 2 to 4 hours most of the organisms are clearly bacillus shaped There are also seen thick forms irregular in outline as if the protoplasm within were distributed irregularly and chain formation is common After 6 to 8 hours incubation the short bacilli and the *coccobacilli* predominate and the long forms are in the minority regularity of morphology is characteristic Cultures which have been

tions and the majority of those isolated from the respiratory tract were first described by Cohen (1909) Scott (1929) described the iridescent aspect of their colonies

The contributions of Pittman (1931) clarified some of the controversial issues Strains of *H influenzae* prevalent in the healthy human respiratory tract were shown to differ from those isolated from persons with *H influenzae* infections It was confirmed that strains cultivated from patients with meningitis bacteremia and pneumonia could be differentiated from nonpathogenic forms by their iridescent growth on Levinthal agar capsules could be demonstrated by special technics Six different types of *H influenzae* were identified by precipitation and agglutination tests the former method demonstrated the presence of the specific soluble substance by its precipitation with homologous diagnostic typing antiserum The 6 types were designated *a* *b* *c*, *d* *e* and *f* type *a* specific substance was shown to be a polysaccharide Virtually all of the meningitic strains were type *b* Dried alcoholic precipitates of the cultures of 2 of the strains reported by Rivers (1921) were also identified as type *b*

The agglutination reaction when incubated at 37 for 2 hours showed the same degree of specificity as the precipitation test However when agglutination was carried out at 47° C for 4 hours (the conventional procedure) there were marked cross reactions among the different types This fact probably explains the failure of the agglutination test in the hands of earlier observers to identify specific types which must have been present in a part of the population More recent studies have shown that the somatic antigens of all 6 types as well as some nonencapsulated strains exhibit immunologic cross reactions Exposure to the higher temperature apparently releases some somatic components The labile capsules of *H influenzae* cannot be identified after such treatment Pittman's study of variation of colonial forms of *H influenzae* demonstrated the process whereby an S strain under artificial cultivation becomes rough the changes observed explained earlier controversies on morphology and some of the failures to identify specific types It is apparent as emphasized by Pittman that the immunologic behavior of the influenza bacillus parallels in a number of respects that of the pneumococcus

During the ensuing 10 years investigations dealt mainly with treatment of pyogenic infections caused by the influenza bacillus The frequency of this organism as a cause of meningitis in children and a mortality rate of over 90 per cent had already been well established *The use of accurate methods for* bacteriologic diagnosis disclosed the fact that *H influenzae* was the most frequent cause of meningitis during years when the incidence of meningococcus infections was low Since type *b* was found to be responsible for almost all cases of influenzal meningitis the production and the use of therapeutic antiserum naturally followed Ward and Fothergill (1932) and Fothergill (1937) produced a therapeutic horse antiserum against type *b* The use of this serum in 220 cases of influenzal meningitis yielded very disappointing results despite its administration twice daily intravenously along with fresh complement and serum intrathecally 84 per cent of the patients died Pittman also produced a therapeutic horse antiserum against type *b* this was used for only a small series but it too was unsuccessful on the whole

In 1939 Alexander using methods learned in the production of pneumococcus rabbit antiserum produced an *H influenzae* antiserum in the rabbit which when applied according to certain principles made it possible to cure 80 per cent of children with *H influenzae* meningitis The principles shown by Dubos to be of importance in pneumococcus vaccine production were applied in an effort to produce a vaccine reflecting the chemical composition of *H influenzae* as it occurs in human infections Attention was focused on attaining optimal encapsulation of the organism It was shown that encapsulated forms of *H influenzae* could be differentiated into types by the capsular swelling phenomenon (Alexander 1939) the technic is comparable with the Neufeld Test devised for typing pneumococci This procedure also proved to be useful for detecting changes in the state of the capsule Study of type *b* *H influenzae* through all phases of the growth cycle in Levinthal broth and agar demonstrated that the capsules are much more labile than those of pneumococci When large inocula are grown for 7 hours the capsules begin to show deterioration and in 24 hours it is difficult to identify them Degeneration of the capsules takes place much more slowly when the inoculum is small, suggesting that enzymes produced by the organisms themselves are re

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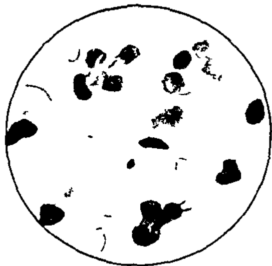


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confirmed by Pittman (1931) offers another point of similarity to pneumococci This trait is characteristic of both pathogenic and non pathogenic varieties of *H influen ae* and therefore is of no differential value

Antigenic Structure

The antigenic pattern of encapsulated pathogenic varieties of *H influen ae* types *a b c d e* and *f* has been shown to resemble that of type specific pneumococci a specific soluble substance is produced by each type and is concentrated in the capsule as well Goebel (reported by Pittman 1931) concluded that type *a* specific substance is a polysac charide Dingle and Fothergill (1939) re ported the polysaccharide nature of type *b* specific sub tance MacPherson et al (1946) agreed on the polysaccharide nature of types *a* and *b* and reported this to be true for types *c d* and *f* as well The specific substance re sponsible for type specificity has been shown by Zamenhof and Leidy (1954) not to be a polysaccharide in types *a b* and *c* but a form of polysugarpho phates Type *b* substance has been shown to be polyribophosphate by Za menhof et al (1953) The type of a given strain of *H influen ae* may be established by capsular swelling agglutination of organisms or precipitation of the specific soluble sub stance with diagnostic typing serum The type specific antigen is responsible for these 3 re actions and also for stimulating the production of protective antibody as well

It is of great interest that 3 types of *H in fluen ae* are related immunologically to certain types of pneumococci (Chapman and Osborne 1942 Alexander et al 1946) Table 45 shows the cross reactions as evidenced by capsular swelling between the type specific antigens of *H influen ae* and pneumococci

The characteristics of the somatic antigens are less well known Platt (1939) isolated 2 proteins a P substance making up the mass of the protein which requires destruc tion of the organism for its liberation and an M substance which is labile small in amount and apparently a surface antigen since it is freed from the intact organism by washing with saline The M substance is toxic for animals and is common to all strains whereas P substance is nontoxic and differs among strains Dubos (1941) obtained from an R derivative of type *b H influenzae* which had

Table 45 Cross Reactions Between Polysaccharides of Pneumococci and Type Specific Antigens of Influenza Bacilli*

Pneumococcus	*H influen ae*
6 Sub group	Type a
6 Sub group	Type b
11 †	Type c
15 A †	Type b
29 Sub group	Type b
35 B †	Type b

* Alexander H E Leidy G and MacPherson C 1946 Production of types a b c d e and f *H influ en ae* antibody for diagnostic and therapeutic pur po es Journal of Immunology 54 207

† Not hitherto recorded

high toxigenic power an antigen lethal for rabbits in 0 1 mg doses immunization of rabbits resulted in resistance to 50 M L D of this substance The toxic substance is be lieved to be an endotoxin

Studies on the somatic antigens of the in fluenza bacillus group by immunochemical procedures suggest the presence of a common antigen in all types of encapsulated *H in fluen ae* and in some nontypable varieties However recent investigations using trans formation technics suggest important differ ences The differences in the antigenic com ponents of *H influen ae H parainfluen ae* and *H suis* demonstrated by immunochemi cal methods may be better understood by the use of the transformation technics

Variation

When cultures of encapsulated strains of *H influen ae* are sparsely seeded on a trans parent medium (Levinthal or Fildes agar) 2 kinds of colonies appear as first reported by Pittman (1931) Almost all of the colonies are opaque and large after 18 hours growth When viewed in obliquely transmitted light they show a characteristic iridescence At times there are present at least one or more colonies which fail to show iridescence they are smaller bluish in color and transparent Transfer of one of the latter colonies to Levinthal broth yields a culture with charac teristics differing from the original no specific soluble substance is detectable capsular swell ing cannot be demonstrated and when the broth inoculum is again seeded on Levinthal agar no iridescent colonies are seen These

growing for 24 hours contain a large amount of amorphous debris and the predominant recognizable form is the minute short, poorly stained coccobacillus, giving the impression that only a part of the organism takes the stain Evidence of autolysis becomes increasingly apparent after 12 hours At first the organisms take the stain less readily later amorphous debris is prominent indicating that the organisms have disintegrated Inoculation of a Levinthal agar plate with 0 5 cc of an 18 hour Levinthal broth culture results in growth in 3 to 4 hours Iridescence is visible by obliquely transmitted light within 4 to 6 hours this quality becomes more striking during the next 2 hours and subsequently starts to decrease After 24 hours the iridescent quality is absent Paralleling this phenomenon the capsules disappear, and the organisms disintegrate There is reason to believe that these 3 changes which occur simultaneously are the result of liberation of enzymes by the bacteria When a much smaller inoculum is used to seed Levinthal agar (a 2 mm loop of 18 hour Levinthal broth culture) maximum iridescence is seen in 18 hours the capsules are well preserved and evidence of autolysis of organisms is absent at that time Apparently a longer period is required for this smaller population to produce sufficient autolyzing enzymes In Levinthal broth the changes in morphology are similar but less pleomorphism is seen and autolysis proceeds more slowly

Cultivation and Biochemical Characteristics

Growth from pathologic fluids may be obtained on blood agar or broth at pH 7 6 somewhat better on chocolate agar Optimal growth takes place in media in which the contents of the red cells are liberated either by heat as in Levinthal (1922) or by peptic digestion as in Fildes (1920) Both of these media have the additional advantage of transparency and therefore are more suitable for the study of the characteristics of individual colonies The presence or the absence of iridescence can be studied also by viewing the growth on the surface of Levinthal or Fildes agar in obliquely transmitted light Growth on these media is influenced by pH and availability of oxygen The optimal pH is 7 6 Increased aeration by frequent agitation or by use of shallow layers of broth enhances growth

The broth we have found most satisfactory is a further modification of the Pittman (1931) changes in Levinthal broth It is made by mixing 1 part of Levinthal stock with 3 parts of neopeptone broth (Lenert and Hobby, 1947) ' Levinthal stock is prepared as follows brain heart infusion broth (Difco) made according to directions on the bottle is heated to vigorous boiling and sterile defibrinated horse blood is added to make a final concentration of 10 per cent The mixture is filtered through Whatman filter paper No 12 and the clear filtrate is sterilized by Seitz filtration

Levinthal agar is made by adding 1 part of sterile ' Levinthal stock" to 1 part of melted agar [45 Gm Proteose Agar No 3 (Difco) plus 15 Gm Bacto agar per liter of water]

Identification of the nontypable influenza bacillus depends upon the need for certain growth factors X and V Lwoff and Lwoff (1937) have shown that X factor acts physiologically as hemin and that V factor can be replaced by coenzyme 1 or coenzyme 2 Subsequently Schlenk and Gingrich (1942) reported that nicotinamide nucleoside can also function as V factor

When *H influenzae* is encapsulated as is usually the case in pathologic fluids the type as well as the genus can be diagnosed by direct swelling of the capsules when the organisms are exposed to type specific antisera

Khristensen (1922) reported that *H influenzae* produced acid from proteins He suggested that this fact might be responsible for the diversity of opinion concerning the ability of this organism to ferment carbohydrates In his opinion evidence for fermentation of carbohydrates was lacking There is general agreement, in any event, that this function is of no differential value

Most strains of *H influenzae* produce indole This is true of a larger fraction of encapsulated strains than of the nonencapsulated There are strains in each group which show no indole production it has proved to be too variable a characteristic to aid in the classification of these organisms

One of the most consistent characteristics of *H influenzae* is its ability to reduce nitrates to nitrites Hoagland (1942) used quantitation of this action for measuring growth of *H influenzae*

The solubility of *H influenzae* in bile first described by Sellards and Sturn (1919) and

Rd and also in Ra and Re populations during growth There is no evidence that susceptible cells reproduce their kind they emerge in all experiments when the population reaches the end of the logarithmic period and a density of 2 to 4 x 10^9 cells per ml In the early logarithmic phase it is difficult to demonstrate the presence of any susceptible cells The peak frequency of susceptible cells occurs in the early stationary phase of growth cycle Thereafter the decline in frequency is gradual

In addition to the presence of variants which differ in certain traits from the rest of the population changes involving the population as a whole occur during the growth cycle some of these have been described already In addition to the morphologic changes there is good reason to believe that important chemical changes caused by autolytic enzymes are also taking place There is no direct evidence that antigenicity of the type specific substance is impaired but use of suspensions of *H influenzae* showing some morphologic changes due to autolysis results in the production of a smaller quantity of type specific antibody than that produced from organisms showing no autolysis also the capsules disappear under these changes Moreover the proof that autolytic enzymes of pneumococci render their type specific polysaccharides antigenically inactive in rabbits suggests that the same series of events may occur in *H influenzae* cultures

Toxins and Pathogenicity

A number of authors (Jordan 1927 and Scott 1929) working on strains of *H influenzae* cultivated from patients with influenza during the pandemic of 1918 reported that the injection of some strains into animals was followed by lethal toxic injury However the size of the lethal dose suggests that the toxic effect was due to an endotoxin and not an exotoxin

There is no evidence that *H influenzae* produces a true exotoxin On the other hand the injurious effect caused by what are probably endotoxins may play a significant role in the pathogenesis of severe infections Whether or not the different pathologic potentialities described for some strains depend upon their capacity to produce this material cannot be answered Nor do we have any evidence that antibody to these toxic substances is important in recovery from *H influenzae* infections On the other hand the antigenic importance of the toxic fractions isolated by Platt (1939) and Dubos (1941) warrants further exploration

H influenzae is not naturally pathogenic for any of the smaller animals Multiplication with invasion of the blood does occur in mice injected intraperitoneally with organisms suspended in mucin (Fothergill et al 1937) or when suspensions in brain are introduced intracerebrally (De Torregrossa and Francis 1941) These tests serve a useful purpose for testing efficacy of antibacterial agents but are not adequate for differentiation between pathogenic and nonpathogenic strains

In monkeys Blake and Cecil (1920) reported bronchiolitis and hemorrhagic bronchopneumonia following intratracheal introduction of a culture of a pathogenic influenza bacillus Wollstein (1911) produced meningitis in monkeys by intrathecal inoculation of *H influenzae*

Wright and Ward (1932) described an in vitro test which could distinguish between strains cultivated from spinal fluid and those found in the respiratory tract of many normal subjects the former were uninfluenced by normal rabbit blood the latter were killed All strains which were well preserved in rabbit blood produced a type specific soluble substance

On the other hand the pathogenic potentialities of *H influenzae* for humans is closely related to the presence of a capsule and the elaboration of a specific soluble substance there is reason to believe that this substance exerts an influence on leukocytes not unlike that described for pneumococcus polysaccharides Nonencapsulated *H influenzae* seldom invades the blood at any age whereas type *b H influenzae* is one of the most frequent causes of bacteremia in infancy and childhood The human infections caused by this organism will be described later

Host Range

True *H influenzae* infections occur only in man Closely allied forms occur naturally in animals Gram negative bacilli classified as *H parainfluenzae* have been isolated from cats by Rivers and Bayne Jones (1923) *H hemoglobinophilus* (Friedberger 1903) is present in large numbers in the preputial secretions of dogs *Brucella bronchiseptica*

variants arise spontaneously under what is deemed to be optimal conditions of artificial cultivation Under certain less favorable con ditions the culture is made up predominantly of these variants and the culture is said to have passed from the smooth to the rough phase

The appearance of R cells from type *b H influenzae* has also been demonstrated in the nasopharynx of patients following recovery from influenzal meningitis Evidence that the R cells were derived from type *b* organisms was offered by the genetic marker in the R cells the inherited streptomycin resistant trait which the type *b H influenzae* grown from the spinal fluid and the nasopharynx also exhibited

There are at least 2 kinds of phenomena which are exerting forces responsible for changes in heritable traits of Hemophilus as well as other populations (1) selection of spontaneously occurring mutants which is so well exemplified by emergence of resistance of *H influenzae* to streptomycin (Alexander and Leidy 1947a and 1947b) and induction of new traits by exposure to a DNA derived from cells possessing the character to be intro duced When nontypable *H influenzae* popu lations derived from types *a b d*, or *e* but lacking capsules and ability to produce spe cific soluble substance and iridescent growth on Levinthal agar are exposed to DNA con taining extracts of type *a b c d e* or *f* certain genetic traits of the donors of the DNA will appear in the recipient populations Type specific populations can be changed to a new type by exposure to DNA from a heterologous type Therefore absence of type specificity is not a prerequisite for induction of a genetic change (Alexander and Leidy 1951b) In pop ulations of either typable or nontypable *H in fluenzae* which are sensitive to streptomycin (Alexander and Leidy 1953a) resistance of a high degree (1 000 mcg per ml) can be induced by DNA derived from populations which show the latter degree of resistance to streptomycin When type *b H influenzae* was exposed to DNA extract of type *a* cells an interesting phenomenon occurred type *a* and type *b* antigens could be demonstrated within the same cell designated Sab (Alexander and Leidy, 1953b) The DNA extract of Sab cells could induce the ab trait in R cells derived from type *d* and individual type *a* and type *b* traits in other cells These data have been in terpreted as evidence of linkage of the heredity determinants of types *a* and *b* Thus the DNA s of *H influenzae* have been shown to function as heredity determinants in vitro

Whether this phenomenon occurs in vivo is not known at present If it can be shown to operate in vivo, the large reservoir of non typable *H influenzae* found in the respiratory tracts of individuals of all ages may prove to be of greater significance than is now appre ciated

In the Hemophilus system in populations known to contain susceptible cells the reaction between the recipient cell and DNA is vir tually immediate and does not require growth However even under the best conditions ac cording to our present knowledge only a very small proportion of the population exposed is susceptible to induction of a genetic change The factors which influence the susceptibility or the competence of cells has assumed great significance

The evidence suggests that in a given popu lation the size of the proportion susceptible to different heredity determinants is the same (Alexander and Leidy 1954) When a popu lation is exposed to mixtures of two different DNA's 75 per cent of the type *b* DNA and 25 per cent of DNA of type *c* 75 per cent of the transformed cells will be type *b* and 25 per cent type *c* Moreover, the action of one DNA previously can completely exclude a DNA added within 15 minutes

Study of factors which influence the pro portion of a population transformed have re vealed 3 (Alexander and Leidy 1954)

1 Type of Origin of Recipient Cells Type *a* exhibits the lowest frequency, about 1 10 000 000 and type *d* the highest approxi mately 1 per 1 000 cells exposed This type specific property which controls the frequency of susceptible cells is an inherited trait re peated change to a heterologous type shows no influence on the incidence of these cells

2 Concentration of DNA Within certain limits increase in the concentration of DNA controlling streptomycin resistance can in crease the size of the proportion of cells in which streptomycin resistance can be induced This result is to be expected if a single unit or molecule is the cause of transformation However 100 fold increases in concentrations greater than 10^{-1} μg per ml have not induced streptomycin resistance in a higher proportion of cells

3 Phase of Growth Cycle Predictable fluctuations in frequency of induced heritabl changes have been demonstrated in Rb and

bilaterally The severe *H influen ae* infections found with greatest frequency are meningitis obstructive infections of the respiratory tract pyarthrosis pneumonia and empyema These clinical patterns are de cribed in detail else where (Alexander 1942) While most of these infections have been reported in adults they occur only rarely

Diagnosis

The importance of severe *H influen ae* in fections in infants and children may be under stood from their frequency and potentially high mortality rates Prior to 1938 meningitis the commonest of the e was almost uniformly fatal over 90 per cent died Moreover except in years when meningococcus meningitis oc curs in epidemic proportions *H influen ae* is the most frequent cause of meningitis The clinical signs of influenzal meningitis do not differ from those due to other varieties of bac terial meningitis Therefore etiologic diag nosis depends entirely upon a bacteriologic identification This statement is also true for *H influen ae* pneumonia empyema pyarthro- sis and other less frequently occurring clinical patterns such as ethmoidal sinusitis with peri orbital cellulitis and edema pericarditis etc

However the situation is quite different when *H influenzae* type *b* is responsible for an obstructive laryngeal infection The illness presents a characteristic history and the pa tient a characteristic appearance

To date almost all these patients at the Babies Hospital have been 2 years of age or older The onset is sudden and the course ful minating The entire length of acute illness in those cases which end fatally is less than 24 hours Mild fever and difficulty in swallowing and complaint of sore throat on the part of the older children make their appearance in the course of an apparently innocuous infec tion of the upper respiratory tract Dyspnea starts abruptly and increases within a few hours to such a degree as to make hospitali zation and tracheotomy imperative The char acteristic picture is that of a prostrated child with dyspnea due to laryngeal obstruction Phonation is unimpaired The temperature is high On examination of the pharynx there is diffuse erythema often with evident edema and when the tongue is pressed downward the enlarged red misshapen edematous epiglottis is seen easily The obstructive syndrome de

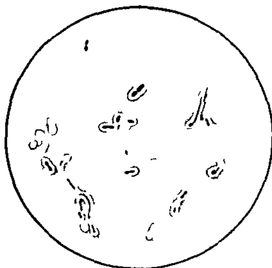


Fig 48 Typing of *H influen ae* by capsular swelling with diagnostic typing sera × 1050 (Alexander H E 1934 Treatment of Haemophilus influenzae infections and of meningococcus and pneumococcic meningitis American Journal of Di eases of Children 66 172 187)

scribed has been found in our experience only in association with *H influen ae* infection therefore when ob erved it suggests such in fection immediately Bacteremia appears to be a constant feature

When organisms are sufficiently numerous in biologic fluids to be seen on stained smear pecies and type may be identified by cap sular swelling (Fig 48) or precipitin test within a few minutes The capsular swelling test is the simpler procedure

A 3 mm loopful of diagnostic typing anti serum is mixed on a cover slip with an equal quantity of pathologic fluid Enough methyl ene blue is added to color the drop lightly and the preparation is inverted on a hollow ground slide and sealed with oil The cover slip may be inverted on a flat slide

The precipitin test can also provide imme diate diagnosis when organisms are numerous when the infection is so mild that bacteria can not be demonstrated microscopically this pro cedure is seldom positive The technic is as follows

In a small precipitin tube (50 x 6 mm) a 1-cm column of fluid to be tested is carefully

plays the same role in dogs and some other animals as *H influenzae* plays in man but its physiology differs much from that of *H influenzae* The role of *H suis* in swine may be quite comparable with *H influenzae* in man, but here too the characteristics of the 2 organisms differ significantly The resemblance of function of these 3 apparently different organisms in 3 animal species raises the question of influence of host on physiology of an organism (See Bergey, 1957 for Hemophilus species in other animals)

Ecology

It is seldom possible to assign a primary pathogenic role to nonencapsulated *H influenzae* in any age group In young infants it occasionally causes meningitis or pneumonia accompanied by bacteremia recoveries from these infections have occurred after the administration of sulfadiazine alone Epidemic conjunctivitis has been ascribed to an organism labeled Koch Weeks bacillus' (Koch 1887 and Weeks, 1887) *H aegyptius* which is indistinguishable by ordinary criteria from nontypable *H influenzae* Subacute bacterial endocarditis is at times caused by *H influenzae* both in children and adults (Rose 1941) The role which this organism plays in chronic lung infections and in some acute lung infections, when the blood cultures are sterile and no other bacterial pathogens are demonstrable is a controversial question which requires more evidence to answer According to some opinions, the influenza bacillus has a destructive action on bronchial epithelium (Zinneman 1943)

There is a striking difference between adults and children in the pathologic potentialities of encapsulated *H influenzae* as a primary pyogenic agent The adult appears to possess effective resistance to it The explanation for this difference is discussed under Immunity However there is reason to believe that should the normal defense mechanism be altered as has been shown to occur in severe pandemics of influenza a greater prevalence of severe *H influenzae* infections in adults is to be expected

In an attempt to learn something of the ecology of this organism unselected patients admitted to the Babies Hospital were studied for the incidence of *H influenzae* (Alexander, 1943a) Some form of *H influenzae* was isolated from about 30 per cent of the children of these strains 19 per cent produced iridescent growth and were therefore typable and approximately 80 per cent of these typabl strains were type *b* Type *b* was the cause of virtually all severe *H influenzae* infections regardless of the clinical type a very occasional case was due to types *a*, *e* or *f* On the other hand, it was found that type *b* causes mild infections as frequently as severe ones A small per cent of children without signs of infection at the time the cultures were made also harbored type *b H influenzae* in the respiratory tract History of recent infection in many of these subjects suggested that they had recovered spontaneously from an infection with this organism Johnson and Fousek (1943) have studied the spread of these infections

Good et al (1943) have reported the occurrence of *H influenzae* in members of families of children who develop meningitis A large fraction of siblings harbor the organism, among the adults the mother is the only member who shows their presence with any frequency Our bacteriologic study of patients after recovery from severe type *b H influenzae* infections treated by any of the available effective agents shows that this organism persists in the nasopharynx for long periods after its elimination from the spinal fluid

About 50 per cent of normal young adults (medical students) were found to harbor nontypable *H influenzae* in the respiratory tract during all but the summer months

Clinical Patterns

It is evident that type *b H influenzae* enters by way of the respiratory tract where in most children it produces a nasopharyngitis usually with some fever There is reason to believe that many overcome the infection spontaneously Others develop sinusitis or otitis media Any portion of the lower respiratory tract may be involved, from the epiglottis and surrounding structures to the alveoli From these foci in various parts of the respiratory tract, invasion of the blood stream occurs not infrequently The meninges and the joints are sites of predilection for localization Rarely pericarditis or subcutaneous abscesses result Recently, we have seen a severe cellulitis involving the submental and anterior cervical regions

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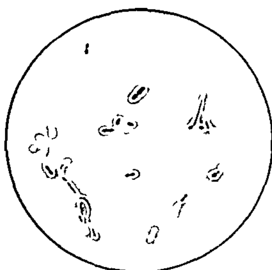


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In a small precipitin tube (50 x 6 mm) a 1-cm column of fluid to be tested is carefully

layered by a fine capillary pipette on an equal column of diagnostic rabbit antiserum A precipitate in the form of a white ring at the interface represents a *positive test* The antiserum used and the pathologic fluid must be perfectly clear Speed of formation of the ring varies with the concentration of specific polysaccharide in the fluid immediate appearance of the ring *denotes high concentration* This time factor may be used as an index of severity of infection of the blood or the spinal fluid

These procedures may be used for immediate diagnosis of *H influenzae* in spinal fluid middle ear or joint exudate or empyema fluid a concentrated suspension of nasopharyngeal mucus from patients with obstructive laryngitis or pneumonia *due to this organism may* reveal its presence when swelling of the capsule is demonstrated The specimen of mucus is collected on a small cotton swab (Alexander et al 1941) passed through the nares to the posterior pharyngeal wall where it is allowed to remain for several seconds to collect mucus at the bedside the swab *is placed in a small* tube containing 0 2 cc of sterile broth

Identification of encapsulated *H influenzae* in cultures from the blood or other fluid may be made after incubation usually for 18 hours by demonstration of capsular swelling with type specific diagnostic antiserum When the latter test is negative Levinthal agar is inoculated to test for *iridescence of growth* If neither capsular swelling nor iridescent quality of growth is demonstrable diagnosis of *H influenzae* must depend on requirement of both X and V factors for growth

Differentiation of Members of Influenza Bacillus Group

Requirements for Diagnosis *Gram negative aerobic, nonsporebearing nonmotile bacilli requiring hemin coenzyme or both for growth* The differential features necessary for diagnosing individual members of the group are outlined in Table 46

Examination of X and V Factor Requirements of Unknown Organisms For this purpose media must be available for testing the separate and combined action of X and V factors

X Factor Medium *Equal parts of* Levinthal stock (used in Levinthal agar) autoclaved 15 minutes at 20 pounds pressure and melted Proteose Agar No 3 (Difco) (45 Gm per liter plus Bacto Agar 15 Gm per liter) X factor is stable and can withstand this treatment V factor is destroyed

V Factor Medium One part of yeast extract to 9 parts of melted *Proteose No 3 Agar* (Difco) Yeast extract can be prepared by the following method modified from Thjotta and Avery (1921) Emulsify 100 Gm of powdered brewer s yeast in 400 cc of distilled water *Adjust pH to about 4 6 boil for 10 minutes*

TABLE 46 *DIFFERENTIAL CHARACTERISTICS OF INFLUENZA BACILLUS GROUP*

	Growth Factors X	Growth Factors V	Iridescence	Capsules	Hemolysis
H influenzae					
Typable *a f*	+	+	+	+	0
Nontypable	+	+	0	0	0
H aegyptius	+	+	0	0	0
H parainfluenzae					
Typable	0	+	+	+	0
Nontypable	0	+	0	0	0
H hemolyticus	+	+	?	?	+
H parahemolyticus	0	+	?	?	+
H suis					
Typable	?	?	+	+	0
Nontypable	?	?	0	0	0
H parasuis					
Typable	0	+	+	+	0
Nontypable	0	+	0	0	0
H hemoglobinophilus	+	0	?	?	0

? = Not determined

Filter emulsion through filter paper adjust filtrate to pH 7 0 and filter through Seitz filter to sterilize Transfer to a sterile container fitted with a glass stopper and seal with sterile petroleum jelly

COMBINED X AND V FACTORS One part of yeast extract to 9 parts of X factor medium

Media are distributed in 3 cc quantities in tubes (100 x 13 mm) and slanted

Cultures to be tested are grown on Levinthal agar a loopful is suspended in 0 2 cc physiologic saline just before inoculating the separate factor media

Pure hemin and coenzyme may also be used as X and V factors

Tickett and Stewart (1953) report that *H influenzae* and *H parainfluenzae* can be distinguished by their satellite formation about growth of catalase positive and catalase negative organisms

Classification of Influenza Bacillus Group According to X and V Factor Requirements

REQUIRE X AND V FACTORS FOR GROWTH

1 *H influenzae*

A Typable potentially pathogenic strains are encapsulated They are classifiable into 6 specific types by swelling of the capsule or precipitation of the soluble specific substance by specific antibody A characteristic iridescent growth is produced on Levinthal agar

B Nontypable nonirridescent nonencapsulated seldom pathogenic organisms cannot be differentiated from encapsulated *H influenzae* on blood agar by morphology of individual members or their colonies Moreover they also require X and V factors Their failure to produce iridescent growth on Levinthal agar identifies them as nontypable nonencapsulated variety

2 *H hemolyticus* Production of beta hemolysis in blood agar distinguishes this organism from *H influenzae* Except for its rare occurrence as a cause of subacute bacterial endocarditis no pathogenic role is recognized It is found frequently in the normal nasopharynx

V FACTOR BUT NOT X IS REQUIRED FOR GROWTH

1 *H parainfluenzae* Human pathogenicity appears to be limited to subacute bacterial endocarditis It is considered as a normal inhabitant of the human nasopharynx Individual organisms are morphologically similar to *H influenzae* except for greater regularity of form and less evidence of autolysis Colonies on blood agar are indistinguishable from those of *H influenzae* This class includes two groups

A Encapsulated *H parainfluenzae* (Lenert and Alexander unpublished) Capsular swelling can be demonstrated with type specific rabbit antiserum and iridescent growth can be demonstrated on Levinthal agar There appears to be more than one type

B Nonencapsulated *H parainfluenzae* Growth is noniridescent and capsular swelling cannot be demonstrated

2 *H parahemolyticus* Production of beta hemolysis distinguishes this organism from *H parainfluenzae* Human pathogenicity is not unlike that described for *H hemolyticus* which requires both X and V factors

H suis Essential growth needs have not been defined This organism reacts synergistically with the virus of swine influenza in the natural and experimental disease of hogs Human pathogenicity is unknown Morphologically this organism does not differ significantly from *H influenzae* and *H parainfluenzae* While Levinthal broth and chocolate agar provide better growth than blood agar growth of most strains on these media is poor Shope reported that X and V factors are essential for growth but study of a number of strains obtained from Dr Shope shows that some do not require X factor In our experience the best growth has been obtained on a modified Levinthal agar with the addition of 5 per cent horse plasma or 10 per cent yeast extract (fresh)

Study of 8 strains revealed 2 different groups

1 Those producing iridescent growth on modified Levinthal medium Capsular swelling occurs on exposure to homologous rabbit antibody Use of antisera produced against 3 iridescent strains failed to reveal immunologic differences among 5 strains No immunologic relationship was demonstrated between the type specific antigens of *H suis* and *H influenzae*

2 Those showing noniridescent growth demonstrated no type specific characteristics

REQUIRE X FACTOR AND NOT V

H hemoglobinophilus is the only known representative of this group Human pathogenicity has not been recognized Friedberger (1903) first described *H hemoglobinophilus* in chronic purulent exudate from the preputial sac in dogs

Studies on the specificity of the DNA controlling streptomycin resistance in *H influenzae* *H parainfluenzae* and *H suis* suggest that the relationship between a given recipient population and the donor of the transforming DNA may be reflected in the ratio of the

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Typable *a f*	+	+	+	+	0
Nontypable	+	+	0	0	0
H aegyptius	+	+	0	0	0
H parainfluenzae					
Typable	0	+	+	+	0
Nontypable	0	+	0	0	0
H hemolyticus	+	+	?	?	+
H parahemolyticus	0	+	?	?	+
H suis					
Typable	?	?	+	+	0
Nontypable	?	?	0	0	0
H parasuis					
Typable	0	+	+	+	0
Nontypable	0	+	0	0	0
H hemoglobinophilus	+	0	?	?	0

? = Not determined

Because complement was found infrequently in the spinal fluid of patients with influenzal meningitis (Fothergill 1935), administration of fresh human serum along with horse antiserum by the intrathecal route was recommended at one time However, the therapeutic results were disappointing

Investigations on the production the measurement and the u e of rabbit type *b H in fluen ae* antibody indicate that immunity to *H influen ae* may not differ significantly from immunity to pneumococci The protective element in type *b H influen ae* rabbit anti erum is the anticarbohydrate antibody Quantitative chemical methods originally developed for measuring anticarbohydrate antibody in pneumococcus antisera are applicable to *H influen ae* antibody Type *b* rabbit antiserum administered only by the intravenous route has proved to be highly efficacious for the treatment of influenzal meningitis without the use of complement Phagocytosis of organisms by leukocytes in the spinal fluid is apparent during treatment with this antiserum Th adequacy of dose of pecific antibody for a given patient may be determined by the ability of hi serum to produce capsular swelling of the organism (Alexander et al 1942) or by his cutaneous reaction (Dingle and Seidman 1941) to a 1/10 000 dilution of specific polysaccharide As in the Francis test (Francis 1933) for excess of humoral antipneumococcus antibody the skin test with influenzal polysaccharide is positive when the circulating blood contains a demonstrable quantity of th specific type *b* anticarbohydrate

Treatment

Prior to 1938 the mortality in patients with influenzal meningitis was close to 100 p r cent Today there are a number of effective therapeutic agents type specific rabbit antiserum (Alexander and Leidy 1942) streptomycin tetracyclines chloramphenicol and polymyxin The location the duration and the severity of infection govern the selection of agents in a given patient In febrile upper respiratory tract infection with early otitis media or sinusitis in obstructive epiglottitis and in pneumonia prompt recovery has occurred following use of sulfonamides alone However the risk of therapeutic failure is virtually eliminated by the use of an additional agent tetracyclines or chloramphenicol In the more serious infections meningitis pyarthrosis with or without osteomyelitis empyema or pericarditis the use of two agents is clearly indicated

Sufficient data are now available for a comparison of 3 different therapeutic programs in meningitis (1) type specific rabbit antiserum and sulfonamides (2) streptomycin and sulfadiazine and (3) chloramphenicol and sulfadiazine (Alexander, 1956) Any of the 3 can be expected to cure virtually 100 per cent of the patients who are treated early in the course of the disease It has not been possible to show a significant difference in the efficacy of these 3 pairs of agents Specific antiserum is eldom used today because of possible serum sickness and cost We have discontinu d streptomycin therapy because of frequency of vestibular dysfunction and occasional deafness The ease with which optimal concentrations can be maintained in the cerebrospinal fluid by either the oral or the parenteral routes and the rarity of injurious effects make the combined action of chloramphenicol and sulfadiazine the treatment of choice for meningitis When the oral route is used for chloramphenicol 200 mg /Kg is given daily in 4 doses (not to exceed 3 Gm) in children when parenteral administration is necessary 100 mg /Kg (not to exceed 2 Gm) is given each 24 hours in 3 doses for 7 days The dose in adults should not exceed 4 Gm orally or 3 Gm parenterally Gantrisin or other sulfonamides are also given for 7 days in a dose sufficient to maintain 10 mg per cent

H DUCREYI

This organism was first described by Ducrey (1890) in the purulent discharge from the venereal disease soft chancre or chancroid Proof of the agency of *H ducreyi* in this infection was furnished by the investigations of Greenblatt (1938) Recognition of this organism as the etiologic agent is important since the disease occurs frequently and may be confused with syphilitic chancre Moreover prompt recovery follows the use of sulfonamides

The morphology of the organisms in local lesions is characteristic chains of small gram negative bacilli occur in strands Cultivation proved to be so difficult that very few pure cultures were obtained until Teague and Dei

number of cells transformed by a heterologous DNA to that induced by a homologous DNA. The frequency of transformation is lower when the DNA originates from heterologous species (Leidy et al., 1956).

Pittman and Davis (1950) have reported evidence which they interpret as showing a clear enough difference between *H. influenzae* and Koch-Weeks bacillus to warrant classification as a separate species *H. aegyptius*. Unpublished data of Leidy and Alexander show that the size of the proportion of cells in *H. aegyptius* populations in which streptomycin resistance is induced by a DNA derived from resistant *H. influenzae* cells is not significantly different from the proportion in which this trait is induced after exposure to DNA derived from *H. aegyptius*, and vice versa. If this result can be interpreted as a close relationship between recipient and donor cells, it may provide a more basic criterion for classification.

Immunity

The striking relationship between age and incidence of influenzal meningitis has been well established by Rivers (1922) and by Fothergill and Wright (1933). The latter authors demonstrated a close correlation between the bactericidal power of the blood of subjects in a given age group and their susceptibility to *H. influenzae* meningitis. In children aged 2 months to 3 years, in whom the incidence is highest, the blood of subjects collected at random shows only a feeble bactericidal capacity toward this organism, whereas older persons have a relatively efficient lethal action and possess at the same time an appreciable immunity to infections caused by *H. influenzae* (Fig. 49). Over 80 per cent of the cases of meningitis occurred in the former age period. Our experience is *quite comparable*.

There is reason to believe that two different immune mechanisms contribute to the bactericidal effect of blood of immune subjects—complement bacteriolysis and phagocytosis. Ward and Wright (1932) reported data in support of the first, and evidence which suggested that the *H. influenzae* antibody responsible was anticarbohydrate (Wright and Ward, 1932).

Their findings focused attention on the importance of complement in the process. Dingle et al. (1938) confirmed the need for complement in the bactericidal action and presented evidence that the animal species from which the complement originated is of importance.

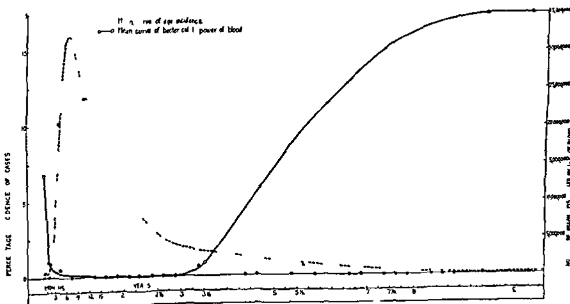


FIG. 49. The relation of age incidence of influenzal meningitis to the bactericidal power of human blood at different ages against a smooth meningeal strain of *H. influenzae*. (Fothergill, L. D., and Wright, J. 1933. Influenzal meningitis. The relation of age incidence to the bactericidal power of blood against causal organism. Journal of Immunology, 24, 281.)

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MORAXELLA LACUNATA

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pleomorphic longer and thread shaped In liquid media a ropy mucoid mass results from growth of old cultures Electron micrographs of the organism reveal a central mass of dense material surrounded by a wide clearer zone which is said to contain antigen

The organism is gram negative staining best when the counterstain is left on for 2 minutes Capsules may be demonstrated by special technic and bipolar metachromatic granules said to represent uneven distribution of cell lipid can be shown by staining with Toluidin blue as suggested by Bordet (Sauer 1957)

Cultivation and Biochemical Reactions

Primary isolation is obtained best on complex media such as that originally used by Bordet and Gengou consisting of a potato blood agar glycerol mixture or some modification of it It should contain at least 15 per cent blood Charcoal may be used to replace blood in certain agar media (Pollock 1947) because it like albumin neutralizes toxic fatty acids which inhibit growth The optimal temperature for growth is from 35 to 37 C

A solid medium which has proved to be satisfactory is prepared as follows

1 Bordet Gengou Agar Base dehydrated Bacto (Prepared by the Difco Laboratories Inc Detroit) This base contains the following ingredients per liter

Potato infusion from	125 Gm
NaCl	5 5 Gm
Proteose peptone Difco	10 0 Gm
Bacto-agar	20 0 Gm

2 Solution of 1 per cent glycerol in distilled water

3 Freshly withdrawn (not over 6 hours) defibrinated sheep s blood

A Suspend 4 Gm of the dehydrated agar base in 100 cc of 1 per cent solution of glycerol in distilled water Heat to boiling to dissolve the medium completely Sterilize in autoclave at 15 lbs pressure (121 C) for 20 minute This base may be stored in 100 cc Erlenmeyer flasks in the icebox

B To prepare the final medium heat the base prepared as described above in a water bath until completely liquefied Cool to from 45 to 50 by placing the flask in a water bath and add the blood to make a concentration of from 20 to 25 per cent and pour plates Use plates for cultures which have been prepared within 72 hours

Satisfactory fluid media have been described by Cohen and Wheeler (1946) and by Verwey (1949)

H pertussis produces no gas and does not attack carbohydrates It neither forms indol nor reduces nitrates In litmus milk alkalinity results in 10 to 14 days as compared with 1 to 4 days in the case of *H parapertussis* of *Br bronchiseptica* About 70 per cent of the strains are catalase positive compared with nearly all strains of *H parapertussis* Citrate is not utilized and urea is not split whereas *H parapertussis* can utilize citrate as the sole source of carbon and readily splits urea

Antigenic Relationship

Bordet and Sleeswyk 1910 observed that all recently isolated strains of *H pertussis* agglutinated in a serum prepared against any one of them thus suggesting a single antigenic type Subsequent workers have generally confirmed this observation However when such strains of the organism are maintained on nutrient agar variant forms occur Leslie and Gardner (1931) from a study of 32 apparently mooth strains described 4 different antigenic phases I the virulent S form IV the completely avirulent form and II and III intermediate serologic variants Of 20 recently isolated strains 18 were found to be in Phase I

Some investigators regarded these variations in antigenic relationship as simply changes in the S → R forms in which varying amounts of Phase I antigens remained on the surface of the bacteria others believed that qualitative differences existed with the various phases as did Leslie and Gardner

The nature of the change in the surface antigens of Phase I organisms during subculture is not clear A rearrangement in the proportion of the different antigens a modification of these antigens or the appearance of new ones have all been suggested

Andersen (1953) by observing the resistance of various strains to heating at 100 C described 2 antigens one was thermostabile (O) the other thermolabile (K) He found that all strains of *H pertussis* had a common O antigen but differed in their content and number of K antigen Furthermore Andersen concluded that *H pertussis* *H parapertussis* and *Br bronchisepticus* have common O antigens and produce similar

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The Pertussis Group

HEMOPHILUS PERTUSSIS

Hemophilus pertussis is a gram negative bacillus generally placed in the genus Hemophilus (family of *Parvobacteriaceae*) Primary isolation requires growth factors contained in blood but the rough variant grows readily on ordinary media For this reason and because *H pertussis H parapertussis* and *Brucella bronchisepticus* are so closely related antigenically, a separate genus (Bordetella) has been proposed for them

H pertussis is the cause of whooping cough a highly communicable acute infection of the respiratory tract characterized by spasmodic coughing, usually in paroxysms ending in a forced inspiration (the whoop) and sometimes by vomiting It is prevalent among infants and children and is frequently the cause of serious pulmonary and cerebral complications and even death especially in young infants

History

Little is known about whooping cough until the Middle Ages when Moulton described drugs used in the treatment of ' the Kink a Scottish colloquialism synonymous with fit or paroxysm It was also known as chin cough' derived from the teutonic word *kindhoest* meaning 'child s cough DeBaillau 1578 is credited with the first classic description of the disease (an epidemic in Paris which was called *coqueluche*)

The patient is seen to swell up and as if strangled hold his breath tightly in the middle of his throat For they are without this troublesome coughing for the space of four or five hours at a time, then this paroxysm of coughing returns, now so severe that blood is expelled with force through the nose and through the mouth Most frequently an upset of the stomach follows

One of the earliest American descriptions was that by Benjamin Waterhouse 1822 A comprehensive account of the disease may b found in Lapin's Whooping Cough 1943

The causative organism *H pertussis* was first observed and isolated from an infant by Bordet and Gengou (1906) Chievitz and Meyer 1916, introduced the cough plate method for bacteriologic diagnosis A more practicable and efficient method, now in general use is the nasopharyngeal swab technic (Bradford Day and Berry 1946)

The early ineffectiveness of pertussis vaccine was explained by Leslie and Gardner (1931) whose work stressed the changes in antigenic phases that occur during artificial cultivation of the organism

Although the dual etiologic role of a virus was suggested by certain investigators it is now clear that *H pertussis* alone can produce the disease in laboratory animals (Shibley 1934) as well as in man (MacDonald and MacDonald 1933)

Morphology

H pertussis when first isolated, is a small nonmotile ovoid bacillus Its mean length like that of *H parapertussis* or *Br bronchiseptica* is 0 5 μ Cells of original cultures are uniform in size but upon subculture become

Route of Infection	Range of LD50 in Millions of Bacteria
Intraperitoneal	280-800
Intranasal	0 26-15 5
Intracerebral	0 18 2 5

It is perhaps significant that Andersen (1952) found a nontoxic variant after intracerebral inoculation which was highly virulent for mice producing septicemia

Study of toxic preparations may be carried out by intradermal injection into rabbits and 10-day-old mice (Katsampes et al 1942 Andersen 1953)

In man the infection is variable in its course but 3 stages may be observed the catarrhal the paroxysmal and the convalescent each lasting approximately 2 weeks The catarrhal stage begins with coryza sneezing and a mild progressive cough about 10 to 14 days after exposure The organism multiplies rapidly upon the mucus membranes of the respiratory tract and soon produces necrosis of the basilar and midzonal portions of the bronchial epithelium with infiltration of leukocytes As the infection extends to the deeper structures peribronchiolitis and interstitial pneumonia results Intra alveolar and localized suppurative lesions are usually caused by secondary invading organisms Edema and hemorrhage often occur in the lung parenchyma

Obstruction of lower airways by mucous plugs causes atelectasis which along with interstitial pneumonia interferes with oxygenation of the blood and leads to acidosis The resulting anoxia is said to be an important cause of convulsions The exact mechanism responsible for postpertussis encephalitis is not known although there is experimental evidence to support an allergic factor

The heat labile toxin may be responsible for lymphocytosis early necrosis of the bronchial epithelium and certain hemorrhagic manifestations

The possible role of allergy in the pathogenesis of the disease has been suggested by Toomey (1938) Parfentjev (1947) showed that mice and guinea pigs injected with *H pertussis* antigens developed sensitivity to the nucleoprotein complex of the organism Pittman (1951) found that mice infected intranasally with the organism exhibited a high degree of sensitivity to histamine Ehrich et al (1942) showed that injection of a sonic extract of the organism into rabbits resulted in leukocytosis which was almost exclusively a lymphocytosis However when mice recovering from the intranasal infection were injected with a whole sonic extract or with such an extract from which agglutinogen had been removed by absorption leukocytosis resulted due chiefly to a polymorphonuclear increase (Bradford Scherp and Tinker 1956)

Immunity

Permanent immunity appears to follow the natural disease Bacteriologically proved instances of second attacks have been noted but are rare Little or no maternal immunity is passively transferred to the newborn Indeed early infancy is a period of great susceptibility and of highest mortality

The relative importance of humoral and cellular immunity is not known Humoral antibodies as measured by complement fixation agglutination opsono cytophagic and mouse protective tests appear during convalescence from an attack and as a result of immunization and persist for several months Sako (1947) observed immunized infants subsequently subjected to family exposures and compared the attack rate with their humoral agglutinative titers The results indicated a positive correlation as follows

Serum Titers	Number Cases	Percentage Attacked
1 320	149	0
1 160	53	11 3
1 40	91	18 7
0	2	33 3

Among the nonimmunized controls with O titers the attack rate was 89 7 per cent

When tested intradermally with purified agglutinogen the reaction is usually negative in susceptibles and positive in immunes There appears to be a certain agreement between positive skin tests to this agent and significant humoral agglutination titers

Diagnosis

Bacteriologic diagnosis can be made by the cough plate culture or by the nasal swab method (Fig 50) The cough plate is exposed by holding it about 6 inches from the mouth

hemorrhagic toxins but may possess common as well as species specific "K" antigens *H pertussis* apparently possesses more antigens in common with *Br bronchisepticus* than it does with *H parapertussis*

Several antigenic components of the S form of organism are now recognized agglutinogen toxin (heat labile and heat stable), hemagglutins and protective

The agglutinogen (Flosdorf et al, 1940 Smolens and Mudd 1943) is water soluble and can be liberated from the cell by sonic vibration and by acid extraction Injected into rabbits it results in a high titer of agglutinin it removes this antibody from such serum by absorption It is nontoxic

The toxin (Evans and Maitland, 1937) when injected intradermally into rabbits, produces necrosis and is lethal for guinea pigs It is not stable and is destroyed by heating at 55 C When formalinized it is antigenic and its antitoxin neutralizes the toxins of *H pertussis H parapertussis* and *Br bronchiseptica* While produced by all phases of *H pertussis* toxin is most abundant in Phase I organisms and only one tenth as abundant in avirulent strains (Flosdorf and McGuinness 1942) Disruption of the organism gives the best yield of toxin but an appreciable amount is contained in the surface washings of the freshly isolated organisms (Katsampes et al 1942)

The heat stable toxin probably of intracellular origin is only partially destroyed by heating at 100 C for 60 minutes Little is known of its nature and properties

Hemagglutinin (Keogh North and Warburton 1947) can be extracted from the freshly isolated organism Antisera to these extracts specifically neutralize their hemagglutin effect Keogh found that resistance to infection parallels antihemagglutin production but Masry (1952) reported that purified hemagglutin was not protective and Standfast (1951) found no correlation between virulence and hemagglutin

Protective antigens have been described and are the subject of controversial opinion Cruickshank and Freeman (1937) and Eldering (1942) isolated carbohydrate antigens that protected mice while Flosdorf and Kimball (1940) and Smolens and Mudd (1943) considered the protective effect to be associated with the agglutinogen Capsular material of *H pertussis* has no significant protective value (Evans and Adams 1942) Robbins and Pillemer (1950) isolated a purified protective antigen from a watery extract of the bacillus which was not carbohydrate, agglutinogen, toxin or hemagglutin It appears to be generally agreed that the protective antigen is associated with the smooth forms of the organism and is more abundant in young rather than old cultures

Host Range and Pathogenesis

H pertussis is an obligate parasite which survives only a short time outside the human host It survives only a few hours in dried sputum and is killed in 30 minutes by a temperature of from 50° to 55 C It is very sensitive to ultraviolet light and to chemical antiseptics

Transmission is by droplet infection and the human carrier plays a minor role While the organism multiplies rapidly in the mucus membranes of the respiratory tract and invades by continuity, the blood stream and even the middle ear is seldom involved However, the latter often becomes infected with other organisms

Experimental infection can be produced in a number of species including the chimpanzee monkey puppy rabbit, rat mouse ferret and chick embryo (see ed 2 p 539)

From experimental infection it is clear that when Phase I and II *H pertussis* is introduced intra abdominally (rabbit or guinea pig) the results are more toxic than invasive (Leslie and Gardner, 1931) More avirulent forms (Phase III or IV) are from 20 to 30 times less toxic

The intranasal instillation into mice gives rise to a characteristic patchy interstitial pneumonia with leukocyte infiltration proliferation of bronchial epithelium and other changes not unlike that seen in the human disease (Burnett and Timmins 1937 Bradford 1938) This type of experimental infection has made available a useful method for immunologic study (Cooper 1952 Bradford 1956)

The intracerebral route of infection was introduced by Kendrick et al (1947) as a means of testing degrees of immunization in mice Comparative virulence tests according to the route of infection in the mouse were reported by Standfast (1951) as follows

usually in a hospital Hyperimmune human serum (McGuinness 1944) injected intramuscularly in doses of from 20 to 40 cc or the gamma globulin produced from it result in humoral antibody levels which compare favorably with those observed in convalescence from the natural disease An injection of vaccine during the incubation period or early catarrhal stage of the disease may have a beneficial anemnestic response

In vitro various drugs inhibit growth of *H pertussis* according to Wells et al (1950) In micrograms per cc the range of concentrations is streptomycin 0 8 to 80 polymyxin 0 08 to 1 0 chlortetracycline 0 16 to 12 5 chloramphenicol 0 16 to 80 and oxytetracycline 0 2 to 12 5

From clinical trial Booher et al (1951) concluded that chloramphenicol and the tetracyclines are of about equal value when given in dosages of 50 mg per kg of body weight

In another clinical trial Ames et al (1953) tested streptomycin chloramphenicol anti pertussis rabbit serum and hyperimmune human serum Each agent showed some modifying effect but none was of convincing therapeutic value Chloramphenicol appeared to be the agent of choice Rabbit antiserum gave the most rapid clearance of the organism from the nasopharynx Penicillin is effective only against susceptible secondary invaders

Epidemiology

Pertussis exists sporadically and endemically throughout most of the world In the United States epidemics tend to occur at intervals of from 2 to 4 years Although it occurs at all seasons most cases are observed during the early winter months in the Northern States and during the spring months in the South The communicability rate in family exposure is about 90 per cent in schoolroom exposure about 25 to 50 per cent This is high for a bacterial disease and is approximately that of such viral diseases as measles and chickenpox

From 1920 to 1953 the mortality from pertussis in the United States decreased from 12 5 to 0 2 per 100 000 population In 1950 the fatality rate for the nation was 0 93 per cent while in New York City (1951 1954) it was only 0 13 per cent The death rate is higher in rural than in urban areas and is highest in early infancy

The disease occurs at all ages About one half of those infected are under 4 years of age about 10 per cent are under 1 year Sixty four per cent of the deaths are under 1 year of age while 40 per cent occur during the first 5 months of life

Pertussis is unique in that its incidence is significant greater among females especially in the later years of childhood Mortality also is higher among females

Control Measures

Effective control of whooping cough requires early diagnosis proper management of the case and contacts and an adequate program for active immunization Because of the insidious onset and variable course diagnosis is delayed longer than in most other common communicable diseases Early recognition is important because the patient is most infective in the initial stage of the disease Culture facilities should be more widely available and used for this purpose

The patient should be isolated for a period of from 4 to 6 weeks and ideally should show a negative culture before release Susceptible exposed subjects should be isolated for 2 weeks These especially infants should receive passive protection (gamma globulin produced from hyperimmune human serum) injected intramuscularly in doses of 2 to 5 cc The previously vaccinated exposed subjects should be given an additional injection of vaccine which gives an anemnestic response in from 4 to 6 days

An adequate program of active immunization should include all infants The initial course of vaccine is usually started at 3 months of age (or earlier) and consists of 3 or 4 monthly injections of vaccine totaling 12 antigenic units This vaccine is usually combined with diphtheria and tetanus toxoids and apparently is effective in a multiple antigen to which poliomyelitis vaccine is added Booster injections (2 to 3 units each) should be given 1 3 and 5 years after the initial course

Encephalopathy following injection of pertussis vaccine has been reported (Byers and Moll 1948) and some 60 instances have been observed This complication though serious



FIG 50 Nasal culture method showing the inhibiting effect of penicillin on the growth of contaminating organisms (*right*) to facilitate the identification of *Hemophilus pertussis* colonies on the surface of Bordet s medium The control plate is shown on the *left* (Bradford W L Day E and Berry G P 1946 Improvement of the nasopharyngeal swab method of diagnosis in pertussis by use of penicillin Am J Pub Health *36* 468)

of the patient while he coughs The nasal swab is passed through the nasal aperture until it touches the posterior pharyngeal wall Upon withdrawal it is passed through a drop of penicillin previously placed upon the surface of Bordet medium and the inoculation is streaked with a flexible platinum loop After incubation for 2 to 3 days at 36 C the characteristic colonies of *H pertussis* are readily apparent especially in the area where the contaminants are inhibited by the penicillin The organism is identified by staining and by agglutinative reactions with specific antiserum

H parapertussis by this technic gives larger colonies well formed in 48 hours with a darker area of discoloration about them

Agglutinins (often 1 160 or higher) appear during the third week of infection and may persist for several months Because they appear so late they are of little practical value in diagnosis

Skin testing with purified agglutinogen often results in a positive reaction in the immune which is said to correlate with complement fixing antibody titer and with the agglutinative titer So far this skin test has not received wide acceptance

Clinically, a history of exposure a progressive cough that becomes paroxysmal and often ends in vomiting or a forced inspiration (the whoop) are highly indicative of pertussis The total white blood cell count usually increases during the end of the catarrhal stage The leukocytes may number 15 000 to 30 000 per cc reflecting a relative and absolute predominance of lymphocytes

Other conditions which resemble pertussis are spasmodic coughs resulting from infected adenoids or sinuses allergic bronchitis bronchopneumonia atypical virus pneumonia mucoviscidosis and mediastinal lesions Parapertussis resembles mild pertussis and can be differentiated only by culture Infections caused by *H bronchisepticus H influenzae* and *Brucella abortus* have been mistaken for pertussis

TREATMENT

Mild and moderately severe pertussis requires only supportive measures but severe cases require the best of professional care,

urea while none of more than 500 strains of *H pertussis* did

H parapertussis possesses common antigenic fractions with both *H pertussis* and *Brucella bronchiseptica* but is identical with neither (Fig 51) Cross agglutination between *H parapertussis* and *H pertussis* is caused by a common minor antigen (Flosdorf 1942) Each of these 3 species has its own thermostabile surface antigens but all have a common thermostabile O antigen (Andersen 1953) *H parapertussis* produces a toxin similar to but less potent than that of *H pertussis* (Bruckner and Evans 1937)

Experimental infection is easily produced in mice by intranasal inoculation characterized by moderate leukocytosis and pulmonary lesions resembling those produced by *H pertussis* (Bradford and Wold 1939)

The incubation period in man is from 6 to 15 days The onset is similar to that in whooping cough but may be more abrupt The cough is less severe frequently spasmodic and sometimes it resembles that in tracheitis The duration of illness is usually from 1 to 3 weeks and complications are rare although fatal pneumonia has been reported (Zuelzer and Wheeler 1946)

The mildness or the absence of symptoms in certain subjects harboring the organism led Lautrop (1954) to suspect the frequent existence of carriers

Reciprocal immunity with pertussis does not exist and there is no evidence that immunization with pertussis vaccine protects against parapertussis It is not known that an attack of the disease confers lasting immunity although second attacks have not been reported

Treatment is symptomatic The organism is susceptible although to a less degree than *H pertussis* to chloramphenicol and the tetracyclines (Day and Bradford 1952) and one of these agents should be used for therapeutic purposes Because of the usual mildness of the disease active immunization has not as yet been regarded as necessary

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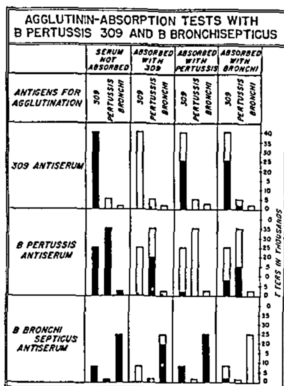


FIG 51 The antigenic relationship of *H parapertussis* (309) with *H pertussis* and with *Brucella bronchiseptica* (Elderling G and Kendrick P 1938 *Bacillus parapertussis* A species resembling both *Bacillus pertussis* and *Bacillus bronchiseptica* but identical with neither J Bact *35* 561 5/2)

should not prevent the routine immunization of infants Caution should be observed during immunization of one who gives a history of frequent convulsions particularly following a previous injection of vaccine Such individuals should receive the vaccine in small doses

HEMOPHILUS PARAPERTUSSIS

Hemophilus parapertussis is a short ovoid gram negative nonmobile bacillus which in many respects resembles *H pertussis* It is the cause of parapertussis an acute respiratory infection resembling mild pertussis from which it can be distinguished only by bacteriologic methods

The organism was first described independently by Elderling and Kendrick (1937) and by Bradford and Slavin (1937) In each instance it was isolated from cases of suspected whooping cough A few strains later recognized as *H parapertussis* were observed in Denmark in 1933 according to Miller The name *Bacillus parapertussis* was suggested by Kendrick (1938), who objected to its inclusion in the hemophilic group

H parapertussis was encountered originally (Bradford and Slavin 1937) in 5 per cent of a consecutive series of positive cultures for *H pertussis* From more than 22 000 diagnostic cultures (1935 1950) Eldering and Kendrick reported (1952) that 19 8 per cent revealed *H pertussis* and 0 5 per cent were positive for *H parapertussis* In Copenhagen Lautrop (1954) found 5 per cent of all cultures taken (representing 16 per cent of all positive ones) positive for *H parapertussis* He observed 256 cases between November, 1950 and March 1952

The demonstration of specific humoral antibodies among children in California (Miller) and in Philadelphia (Flosdorf) indicates the infection to be rather common and easily overlooked In a random sample of routine hospital admissions (531 infants and children in Rochester) 7 1 per cent revealed agglutinins against *H parapertussis* in titers of 1 320 or greater while 34 4 per cent had similar titers against *H pertussis* (Scherp et al 1954)

In schools day nurseries and camps local outbreaks have been observed sometimes occurring with pertussis In family exposures Lautrop (1954) observed the following incidence according to age 0 to 7 years 85 per cent 7 to 17 years 35 per cent adults, 10 per cent

H parapertussis on primary isolation, grows readily on Bordet medium, the colonies appearing within 24 to 48 hours They are smooth round glistening and are surrounded by zones of dark discoloration No report of its primary isolation in bloodless medium is as yet available On subcultures it grows more readily than does *H pertussis* on plain agar and in liquid media suitable for the latter organism

Practically all strains produce catalase (Lautrop 1954) It does not produce indol or hydrogen sulfide nor does it reduce nitrates Milk is made alkaline more readily and to a greater degree than by *H pertussis* All strains examined by Lautrop (1954) split

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The Meningococci

The meningococci are gram negative cocci usually occurring in pairs which form non pigmented translucent colonies and ferment dextrose and maltose with production of acid They are aerobic nonsporulating and non motile They are strict parasites of man and are the cause of several types of infections of which the most important are meningococcemia and meningococcal meningitis The accepted name is *Neisseria meningitidis* although occasionally used synonyms are *Neisseria intracellularis* and *Diplococcus intracellularis meningitidis*

HISTORY

Epidemic cerebrospinal meningitis is undoubtedly an ancient disease but its early history remains unclear because of the difficulty in differentiating it from other syndromes of a similar nature This confusion is exemplified by some of the names used to describe the disease in the early 19th century viz sinking typhus spotted fever and brain fever According to Hirsch (1886) the disease was first recognized in epidemic form in Geneva Switzerland in 1805 by Vieusseux and 1 year later in the United States at Medfield Mass where an outbreak was described by Danielson and Mann During the next 75 years an increasing number of outbreaks of a disease which may be presumed to be epidemic cerebrospinal meningitis on the basis of clinical and epidemiologic data occurred in Western Europe and the United States Military forces particularly those stationed in barracks seemed to be unduly prone to epidemics of the disease By the end of the century outbreaks had been reported from Asia Africa and Australia as well The isolation and the description of the causative organism *Neisseria meningitidis* from spinal fluid of a clinical case by Weichselbaum in 1887 permitted the diagnosis to be made with certainty Since then extensive outbreaks have occurred in many parts of the world A major epidemic occurred in New York City in 1904 and 1905 During World War I the Allied Forces both in England and on the Continent suffered from outbreaks of the disease In the United States in recent years it has tended to occur at irregular intervals There was a severe outbreak in the period of 1928 to 1930 and another during the opening years of World War II 1940 to 1943

With the wide spread application of diagnostic laboratory procedures our knowledge of the disease increased rapidly It was recognized that meningococcal meningitis could occur endemically as well as in the form of epidemics A septicemic form of the disease in which the central nervous system was not involved was recognized and given the name meningococcemia The organism was isolated from the nasopharynges of a significant number of individuals during the course of routine surveys and based on this evidence the concept of the healthy carrier developed

Dopter (1909) was the first to recognize the existence of serologically specific types of

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killed organisms It provides a useful means for the provisional differentiation of the members of the genus *Neisseria* from other microbial organisms which with rare exceptions lack this enzyme

As previously mentioned the possession by meningococcus of an active autolytic enzyme system results in swelling loss of staining properties and in the ultimate disappearance of the cell itself in cultures more than a few hours old This process may be halted by the inactivation of the autolytic enzymes either by heating the culture to 65 C for 30 minutes or by the addition of potassium cyanide or formalin

The resistance of the meningococci to physical and chemical agents is relatively low and these organisms experience considerable difficulty in surviving outside the human body They are quite susceptible to desiccation and are killed by heating to 55 C for 30 minutes or by exposure to any of the common germicides in relatively low dilutions Most strains of meningococci are sensitive to the action of the sulfanomides penicillin and of broad spectrum antibiotics such as the members of the tetracycline series Although fresh strains are sensitive to the action of streptomycin highly resistant forms develop rapidly and in some instances streptomycin dependent strains are the end result

ANTIGENIC STRUCTURE

At least 4 broad serologic groups of meningococci have been recognized on the basis of antigenic differences The original classification of Gordon and Murray (1915) has been followed in its general outline but the use of different terminologies by English French and American workers has caused a considerable amount of confusion The subject has been reviewed recently by Branham (1953) The classification described in Table 47 follows that recommended by a subcommittee of the Nomenclature Committee of the International Association of Microbiologists in 1950 For clarification the terminology in common use in the United States since 1940 is set up in parallel together with the original classification of Gordon and Murray

Of the 4 types originally described by Gordon and Murray I corresponds to Group A II to Group B and IV to Group D It is the general consensus today that their Type III cannot be distinguished from Type I and therefore both are included in Group A Strains falling in this group (A) are the causative agents of the great majority of epidemic outbreaks of the disease Group B has been responsible for the majority of sporadic cases occurring in interepidemic periods Group C originally designated II *alpha* has been isolated chiefly from sporadic cases of the disease Very few strains falling into Group IV have been isolated in the United States There is relatively little crossing over between the 4 groups In addition to these a number of nasopharyngeal strains have been isolated which have not been agglutinated by any of the specific antisera These organisms apparently play no significant role in the causation of disease The production of group transformation has been described by Alexander and Redman (1953)

TABLE 47 GROUPING OF MENINGOCOCCI

RECOMMENDED BY SUBCOMMITTEE 1950	DESIGNATION IN COMMON USE	GORDON AND MURRAY (1915) TYPES
A	I	I III
B	II	II
C	II *alpha*	
D	IV	IV

Group A and C strains are better antigens than those belonging to Group B and long courses of immunization are necessary with the latter in order to produce high titered diagnostic antisera Chemical fractionation (Scherp and Rake 1935 Kabat 1943) has revealed 3 types of antigenic substances A nucleoprotein or P substance is found in all meningococci as well as in other *Neisseria* and in Type III pneumococci This material is apparently responsible for the toxicity of these organisms A polysaccharide or C substance is also found in other members of the *Neisseria* as well as in certain unrelated organisms In addition a polysaccharide specific for Group A meningococci has been identified and purified in the form of the sodium salt of a polysaccharide acid Although not a complete antigen in itself it is presumably responsible for the specific serologic reactions given by Group A Among these are included the capsular swelling and the so called halo reaction This last reaction represents the formation of a ring of precipitate around the individual colonies of the Group A meningococci

meningococcus This work was continued and extended by British and French workers during World War I and laid the firm basis for our present system of classification Serum therapy for meningococcal meningitis was introduced by Jochmann in 1906 and its use was firmly established by the work of Flexner (1913) The era of chemotherapy started in 1939 and today this form of treatment has completely displaced serum therapy The outbreaks of epidemic disease which accompanied World War II gave investigators the opportunity to carry out valuable investigations on the epidemiology the therapy and specific prevention of the disease The recent reviews by Branham (1953) by Goeters (1954) and by Scherp (1955) may be consulted for further reference

MORPHOLOGIC AND BIOCHEMICAL CHARACTERISTICS

The meningococci are gram negative non motile nonsporulating cocci approximately 0 8 microns x 0 6 microns which in body fluids and liquid media at least are often arranged in pairs giving rise to the synonym diplococcus When found in pairs the adjacent sides are usually flattened to produce the typical biscuit or reniform shape Considerable variation occurs in both size and staining properties particularly in older cultures Autolysis is a prominent characteristic of the meningococci and is presumably responsible for the numerous ghost cells seen in older cultures as well as for the marked variations in staining properties The organism is usually described as being nonencapsulated since no capsule has been demonstrated unequivocally by direct capsular stains On the other hand in at least 2 groups of meningococci a capsule may be demonstrated in freshly isolated strains by the application of homologous typing serum

On solid media meningococci give rise to smooth nonpigmented and nonhemolytic colonies Freshly isolated strains produce transparent glistening colonies with a smooth border when grown upon a transparent medium The so called lens effect may be observed in that when distant objects are viewed through the colony they become sharply focused Some strains tend to produce mucoid colonies As the colony ages it tends to become opaque and granular and to lose its smooth border Old laboratory strains frequently give rise to rough colonies

Ever since the publication of Murray s monograph (*1929*) meningococci have been regarded as highly fastidious organisms which require accessory growth factors such as are present in blood, serum and certain vegetable extracts Many media incorporating one or *more of these products have been devised* for the propagation of these organisms However, their definitive growth requirements remain to be determined and more recent work by Frantz (1942), among others suggests that the primary difficulty is not a nutritional deficiency but rather the extreme sensitivity of meningococci to the toxic effects exerted by a variety of amino acids, fatty acids and salts The role of animal proteins and vegetable extracts in culture media appears to be one primarily of absorbing toxic substances so as to permit growth rather than the furnishing of any necessary growth requirement An atmosphere containing 5 to 10 per cent carbon dioxide enhances the growth of most strains of the organism

The meningococci grow best at a temperature of 35 to 37 C At temperatures higher or lower than this, the amount of growth falls off rapidly The organisms are strict aerobes and no growth takes place under strictly anaerobic conditions Blood agar, chocolate agar trypticase soy agar and the starch casein hydrolysate agar of Mueller and Hinton (1941) represent the solid media in common use for the propagation of these organisms Growth in broth (tryptose phosphate veal infusion starch casein hydrolysate) is relatively poor resulting in a granular turbidity and little or no surface growth

The carbohydrate fermentation properties of these organisms are sharply limited Dextrose and maltose are fermented with the production of acid but no gas Lactose sucrose levulose and other sugars are not fermented Indole and hydrogen sulfide are not formed but catalase is present In common with other organisms belonging to the genus *Neisseria* meningococci contain cytochrome oxidase which rapidly oxidizes dimethyl or tetramethyl paraphenylene diamine hydrochloride (McLeod et al 1934) The presence of this oxidase cannot be demonstrated in

acteristic pathologic findings Presumably the endotoxic material released from the bacterial cells as a result of the process of autolysis is responsible for the initiation of the process The vascular system appears to be particularly sensitive to the action of this material and hemorrhagic manifestations are common in the disease In fulminating meningococcemia this hemorrhagic process is particularly noticeable in the skin and in the adrenal cortex Significant involvement of the latter organ gives rise to the so-called Waterhouse Friderichsen syndrome Significant damage to the adrenal gland gives rise to the classic symptomatology of acute adrenal cortical failure and carries with it a grave prognosis

Meningococcal infection of the nasopharynx may set up so mild a reaction in the host that it attracts no notice In other instances an acute inflammatory process may progress to the stage of a purulent rhinitis

Meningococcemia presents the picture of acute sepsis with fever chills malaise and prostration Usually the typical rash can be detected early in the disease This consists of dusky red spots or petechiae of varying sizes up to 15 mm in diameter which involve the skin and the mucous membranes of the body In severe cases the petechiae may assume a purpuric appearance as the result of hemorrhage These may reach a diameter of several centimeters and go on to actual necrosis In mild cases the spots disappear within a few days leaving small brownish areas in their wake histologic examination has revealed that these lesions are due to thrombo embolic involvement of the capillaries Puncture of the lesion frequently demonstrates the presence of meningococci Fulminant infection often give rise to the Waterhouse Friderichsen syndrome characterized by circulatory collapse and shock Diffuse hemorrhage into and necrosis of the adrenal gland are the essential pathologic lesions In the absence of this complication recovery is the general rule if the disease is treated vigorously and promptly

Invasion of the central nervous system is marked by signs and symptoms of meningeal irritation and inflammation Severe headache pain in the posterior aspects of the neck on forward flexion nausea vomiting and often coma are prominent features Physical examination reveals muscular spasm stiff neck exaggerated reflexes and positive Kernig and Brudzinski signs Convulsions and bulging fontanelles are common findings in infants

The meningitis is characterized by an acute inflammatory reaction accompanied by thromboses of the smaller blood vessels and hemorrhage In the later stages marked thickening of the meningeal coverings is a prominent feature as the originally purulent exudate becomes organized Encephalitic involvement is characterized by focal areas of hemorrhage, thrombosis and perivascular infiltration

IMMUNITY

The mechanism of recovery from clinically recognizable meningococcal infections has not been determined During the course of the clinical illness antibody formation may be demonstrated by a variety of technics including agglutination mouse protection and bactericidal tests among others The role that this antibody formed during the course of the illness plays in the recovery of the patient remains uncertain Because of the low morbidity rate which makes it statistically improbable that second attacks will occur and because of the existence of several serologically specific meningococcal groups or types nothing is known concerning the potential immunity conferred by a clinical attack of the disease

There is no reliable method for measuring the susceptibility of an individual to either clinical or subclinical infections with meningococci As is discussed in greater detail in the section entitled Epidemiology man appears to be relatively susceptible to inapparent nasopharyngeal infections with these organisms This is evidenced by the fact that a significant carrier rate of meningococci can be demonstrated in the general population throughout the year Most of these infections are entirely silent although a few may give rise to a local inflammatory process in the nasopharynx On the other hand the resistance of man to clinical infections appears to be relatively high in that cases of meningococcemia and meningococcal meningitis are relatively rare even during epidemic periods It has been shown by Thomas and Dingle (1943) among others that a good proportion of the adult human population possesses bactericidal antibodies against the meningococci which may be dem

when they are inoculated into an agar medium containing an excess of specific antibody Similar reactions have been observed with Groups B and C strains when they are brought into contact with specific antisera These findings suggest the presence of capsular material of a specific serologic nature but this component has not been characterized chemically as yet In the case of Group A antisera at least there appears to be a close correlation between their antipolysaccharide content and their protective ability After absorption of the antiserum with the specific polysaccharide there remains some residual antibody which affords appreciable protection in mice against experimental meningococcal infections (Kabat et al 1945)

Group B meningococci contain a protein *fraction which is common to all members of* the group and is antigenic In addition there is a carbohydrate polypeptide complex (kappa substance) which although not a complete antigen in itself reacts with Group B specific antibodies

Since the early work of Flexner (1907) investigators have recognized the lethal activity of heat killed or autolyzed cultures of the meningococci The fact that such preparations are markedly toxic for laboratory animals has suggested that an endotoxin released by the autolysis of the bacteria is responsible for many of the signs of the disease The endotoxin appears to be relatively nonspecific antigenic and heat stable presumably it is a nucleoprotein

NATURAL HABITAT AND RANGE *OF PATHOGENICITY*

The meningococci are strict parasites of man their natural habitat is the nasopharynx of this species Natural infections are found only in man and the organisms have a relatively low virulence for other species Flexner 1907) was able to produce a picture of meningitis in monkeys following the intraspinal inoculation of a relatively large number of organisms As pointed out by Murray (1929) laboratory animals such as mice guinea pigs and rabbits are susceptible to the inoculation of meningococci only when the infecting dose is so large that it approaches the lethal dose of the heat killed organisms However if the organisms are suspended in 2 to 5 per cent hog gastric mucin (Miller 1933) a relatively small number of organisms suffices to initiate a fatal infection The enhancing effect of the mucin is due presumably to the protective effect it exerts against the body s natural defense mechanisms Although this technic has *been employed in the determination of anti* body levels in sera and of the relative efficacy of drugs this type of infection bears little resemblance to the natural disease as it is seen in man hence its usefulness is limited Embryonated eggs are susceptible to infection following inoculation of the organisms via the yolk sac route and this technic too has been employed for the assay of antibody levels and the therapeutic efficacy of certain drugs There is a considerable variation in virulence between strains of meningococci Old laboratory strains and those which have gone rough are of relatively low virulence as compared with strains freshly isolated from human beings

PATHOGENESIS

Meningococci gain entrance to the human body via the nasopharynx After becoming implanted in this area they may set up a localized inflammatory reaction or they may remain completely quiescent giving rise to no signs or symptoms In a relatively small pro*portion of these infections invasion* of the bloodstream takes place This may remain limited as a simple bacteremia or as the organisms are disseminated through the body metastatic lesions may be set up in various sites such as the skin joints ears lungs and *adrenal glands and, most important of all* the central nervous system Here an inflammatory reaction involving the meninges of both the brain and the spinal cord is the chief finding While it is possible that in some instances organisms spread directly from the nasopharynx into the meninges by *penetrating* through the cribriform plate, it is generally accepted that the central nervous system is invaded from the bloodstream Those factors which govern the ability of the organisms to spread from the nasopharynx into the blood stream and ultimately to reach the *central* nervous system in a small percentage of these cases remain undetermined In most infections it appears that the organisms do not progress farther than the nasopharynx

It is not clear what are the mechanisms by *which the meningococci give rise to the char*

a positive precipitin reaction may be obtained in cases of severe infections in which the organism has been demonstrated in the sediment. Occasionally a positive reaction may be obtained even when no organisms are visible in the smear. The reaction observed is usually specific for the various serologic groups of meningococci and thus affords a rapid method of diagnosis.

Since meningococcal meningitis represents a purulent form of the disease, the spinal fluid shows a predominance of polymorphonuclear leukocytes. The finding of such a fluid in the course of an epidemic should be taken as presumptive evidence of infection due to the meningococcus until proved otherwise. The general inflammatory nature of the meningeal reaction is reflected by the increase of spinal fluid protein and by the abnormal colloidal gold curve. If organisms are present in significant numbers, the spinal fluid sugar level is reduced accordingly, as in the case of other pyogenic forms of meningitis. Nasopharyngeal cultures are of importance since in most cases of meningococcal meningitis and meningococcemia cultures taken from this area yield positive results. In such instances the organisms isolated from the blood or the spinal fluid and from the nasopharynx are almost inevitably of the same group. The culture is taken by means of a bent wire swab with cotton wool on the end. This is inserted carefully behind the uvula and the soft palate so as to avoid contamination from other oral structures, and is rubbed gently over the surface of the posterior nasopharynx. Then the swab is drawn over part of a suitable plate, and the streaking is completed by means of a wire loop. Prompt inoculation and incubation of these plates are of importance; however, if this is impossible, the meningococci may be preserved by inserting the swab into a small amount of sterile horse blood contained in a sterile tube. Under these conditions apparently there is no significant diminution in the number of viable organisms over a period of some hours. If positive, blood and spinal fluid usually yield the organism in pure culture; frequently nasopharyngeal cultures give the same results. Suspicious colonies

TABLE 48. DIFFERENTIAL CHARACTERISTICS OF *Neisseriae*

Organism	Appearance of Colony after 24 Hours Incubation	Growth on Plain Nutrient Agar	Growth at 22° C	Fermentation: Dextrose	Fermentation: Maltose	Fermentation: Sucrose	Other
N. intracellularis	Round, smooth, glistening, translucent, colorless, creamy consistency		−	+	+	−	4 distinct antigenic groups serologically
N. gonorrhea	Similar to *N. intracellularis*. Smaller and more opalescent	−	−	+	−	−	
N. catarrhalis	Smooth, glistening, translucent or firm, somewhat opaque and adherent. May be difficult to emulsify	+	+	−	−	−	Often agglutinates in normal horse serum or saline
N. flavescens	Yellow pigmentation when first isolated; otherwise similar to *N. intracellularis*	+	+	−	−	−	Homogeneous distinct group serologically
N. sicca	Small, somewhat opaque, wrinkled colonies, quite brittle	+	+	+	+	+	Spontaneous agglutination in saline and normal horse serum

onstrated in the test tube The role played by these protective antibodies in limiting the number and the severity of clinical infections remains undetermined However the erratic results obtained with various strains of menin gococci suggest that it does not play a dom inant role Presumably these antibodies are formed in most instances as a result of in apparent infections but this theory remains to be proved

Although there is some degree of positive correlation between the presence of group specific antibodies and the protective value of a given serum (Branham 1940) the fact that absorption with the whole homologous or ganism (as opposed to the specific polysac charide alone) is necessary for the complete removal of the protective effect of the serum suggests that some other material (antigen or haptene) is playing a significant role in the production of protective antibody Pre sumably this protective antibody or antibodies exerts its beneficial effect through promoting phagocytosis by a process of opsonization and through enhancing the bacteriolytic process In this last regard it appears that both group specific antibody and complement play sig nificant roles in the mechanism of the process Although numerous attempts have been made to develop a satisfactory process of active immunization the results in terms of pro tective antibody formation have been incon clusive and in general poor

DIAGNOSIS

The laboratory diagnosis of meningococcal infection is made by the isolation and the identification of the specific organism Blood spinal fluid and nasopharyngeal swabs repre sent the 3 most important materials to be examined for the presence of these organisms Although the technics required for the isola tion and the identification of these organisms are not difficult they do necessitate careful attention to the details of the work Further more a thorough knowledge of the patho genesis of the disease is required in order that cultures may be taken from proper sites at the correct moment

The importance of blood cultures in the diagnosis of meningococcemia is obvious in meningococcal meningitis these are positive in approximately half the cases if they are taken early in the course of the disease Blood taken from the basilic vein is inoculated into both liquid and solid media From 5 to 10 ml of blood is added to 100 ml of tryptose phos phate veal infusion or casein hydrolysate broth Approximately 0 1 ml of blood is spread over the surface of a blood agar choc olate agar or casein hydrolysate plate These are incubated at 35 to 37 C under condi tions of increased humidity and carbon di oxide content (5 to 10%) Cultures should be inspected daily for 7 days before being discarded as negative In cases of overwhelm ing sepsis it is not uncommon to recognize gram negative diplococci in simple blood smears prepared for routine hematologic ex amination Similarly, in such cases where petechiae or a purpuric rash are a prominent feature it is often possible to demonstrate the specific organism in these lesions by means of smear or culture or broth

Spinal fluid is collected by lumbar punc ture in the fourth lumbar interspace The spinal fluid is permitted to drop directly onto the surface of plates of casein hydrolysate, blood agar or chocolate agar medium It is well to inoculate a tube of broth in addition and most authorities recommend the incuba tion of a few ml of spinal fluid collected in a sterile test tube since upon occasion positive results will be obtained in this material upon subinoculation when the other cultures re main negative Finally, approximately 5 ml is collected for physical examination includ ing cell count and smear and in addition immunologic tests A marked polymorphonu clear pleocytosis is the usual finding The fluid is centrifuged and the sediment stained with methylene blue in order to reveal the typical extracellular or intracellular diplococci It is imperative that this examination be made promptly because of the tendency of these organisms to undergo autolysis In those instances where organisms are found in great numbers the addition of a small amount of specific antiserum may result in the demon stration of capsular swelling and the conse quent serologic identification of the causative organism A small amount of the superna tant fluid may be transferred to a small precipitin tube in which it is layered gently over an approximately equal amount of mono valent antimeningococcal serum Frequently

nously plus parenteral fluids to combat vasomotor collapse. Acute congestive failure due primarily to acute interstitial myocarditis is a common complication, and the case fatality rate is high even in the presence of vigorous and sustained treatment.

Serum therapy was introduced by Jochmann in 1906, and its use on a wide scale was stimulated by the report of Flexner in 1913. With the recognition of serologic groups among the meningococci came the development of polyvalent sera for the treatment of the disease with a concomitant improvement in results. Although serum therapy is immunologically sound and the beneficial results obtained unquestioned, it has found little use since 1940 because of the superior efficacy and ease of administration of chemotherapeutic agents such as the sulfonamides and penicillin.

EPIDEMIOLOGY

Man represents the only recognized reservoir of the meningococcus. The portal of entry and exit is the upper respiratory tract, and the means of spread is essentially person to person via air borne droplets or by inanimate objects which are contaminated by the nasopharyngeal secretions of individuals carrying the organisms. The extreme sensitivity of the organism to adverse physical conditions, such as heat and low humidity, means that intimate contact between two individuals is necessary for its spread.

The clinical disease occurs both sporadically and in epidemics (Hirsch, 1886). The latter show some evidence of cyclical occurrences (Gover and Jackson, 1946; Hedrich, 1952) with peaks of high prevalence occurring at 8- to 12-year intervals. The incidence is usually highest in the late winter and early spring months; summer and autumn show a significantly lower incidence of cases. Young children are particularly at risk, with some evidence suggesting a higher incidence in males than in females. The case fatality rates are highest among the young and the aged.

The identification of the causative organism, the discovery of the multiplicity of antigenic types, and the development of effective laboratory methods for the isolation and the identification of the organism have increased our knowledge of the epidemiology of meningococcal infections. Inapparent or subclinical cases far outnumber clinically recognizable cases. Healthy carriers are fairly common in both interepidemic and epidemic periods, yet even in the latter relatively few of the carriers develop clinically recognizable disease. A positive correlation between the carrier rate and the incidence of clinical cases has long been postulated, but careful analyses carried out in World War II (Phair and Schoenbach, 1944; Aycock and Mueller, 1950) have shown that no such relationship exists.

The vast majority of epidemic outbreaks of meningococcal meningitis are due to Group A strains, although small outbreaks due to Group C strains have been reported. Sporadic cases of the clinical disease are usually due to Group B or C strains. Cases due to Group D strains are so rare that the data are inconclusive. These facts are reflected in the carrier rates. In interepidemic periods, Group B strains represent the predominating organism isolated from carriers; in actual fact, Group A strains are marked by their rarity. However, in epidemic periods, strains belonging to this latter group may furnish a significant proportion of the organisms isolated from apparently healthy individuals.

The clinical disease has long been associated with military camps and barracks, and in these, recruits have appeared to suffer far more than seasoned troops. *Immunologic inexperience and susceptibility to excessive fatigue consequent upon the "hardening" process of the raw recruit* have been given as factors *influencing* this difference of incidence, but convincing evidence is lacking. No doubt overcrowding, a situation often found among military and civilian groups during wartime conditions, favors the spread of the organism from one individual to another (Hirsch, 1886). However, even during the course of an epidemic in a closed group, it is difficult to find evidence of spread from one *clinical* case to another. The epidemiology of meningococcemia and meningococcal meningitis will not be clear until some understanding and recognition are obtained of those factors in the host which permit or favor the development of the clinically innocuous carrier state into a full blown case of a dramatic and dangerous disease.

and broth cultures may be subcultured for the identification of the specific organism by means of gram stain fermentation tests and serologic typing The morphologic appearance of the meningococcus on smear has been de scribed These organisms usually ferment maltose and dextrose although the reaction may be delayed and somewhat erratic in the case of freshly isolated strains Sucrose is not fermented For serologic identification sus picious colonies are emulsified in small amounts of saline which usually contain 0 1 per cent potassium cyanide to inhibit the autolytic process The addition of group specific antisera A B or C usually results in the specific identification of the organism by means of the agglutination test Strains iso lated from the nasopharynx during interepi demic periods are frequently untypable Error due to nonspecific agglutination should be guarded against by the use of a normal control serum diluted 1 50 and of a saline suspension of the organism The tube agglutination test has been found to yield reliable results the suspension is shaken at room temperature and incubated at 37 C for 2 hours in a water bath before being examined A final confirm atory examination may be carried out after overnight incubation at 4 C

Growth characteristics fermentation reac tions and serologic tests serve to differentiate other members of the genus *Neisseria* from the meningococci (Table 48) *Neisseria catar rhalis* and *N sicca* are characterized by their ability to grow at room temperature The latter forms rough dry appearing colonies and both species tend to agglutinate spontaneously in saline or normal horse serum Of the vari ous pigmented species of *Neisseria* only *N flavescens* appears to be of any importance since it was isolated from a small epidemic of meningitis in Chicago by Branham (1930) The growth characteristics of the gonococcus closely resemble those of the meningococcus As regards biochemical reactions *N catar rhalis* and *N flavescens* fail to ferment dex trose, maltose and sucrose and the gono coccus ferments only dextrose On the other hand *N sicca* ferments all 3 sugars Agglu tination tests will usually demonstrate the antigenic specificity of these organisms Since all *Neisseria* possess the oxidase which attacks dimethyl or tetramethyl paraphenylene diam ine hydrochloride this reaction is of little diagnostic value except in instances of cul tures taken from the nasopharynx, where it may be helpful in the recognition of organisms presumably falling into the genus *Neisseria*

TREATMENT

Chemotherapy has completely displaced serum therapy in the treatment of meningo coccal infections The drugs of choice are the sulfonamides notably sulfadiazine and sulfi soxazole (Gantrisin) The meningococci are extremely sensitive to the action of these drugs in vitro and this effect is paralleled by the prompt clinical response in the average uncomplicated case if treatment is initiated early in the course of the disease If the pa tient is conscious oral administration of the drug usually will suffice although frequently a priming dose of parenterally administered drug is given at the onset of treatment in order to build up adequate levels in the blood as rapidly as possible In the unconscious or critically ill patient the drug must be admin istered parenterally Due precautions must be taken to maintain a satisfactory renal output to avoid the danger from excessive crystal luria Specific therapy must be maintained for several days after the patient s clinical course has returned to normal

Excellent therapeutic results have been ob tained with penicillin although it is the con sensus that it does not equal the sulfonamides in either effectiveness or in convenience of administration It is common practice today to employ combined therapy with sulfadiazine (or Gantrisin) and penicillin this seems to be unnecessary except in gravely ill patients Since the meningococci are sensitive in vitro to the action of the broad spectrum anti biotics these may be used if the other drugs are unavailable Fortunately the emergence of drug resistant strains during therapy has occurred with negligible frequency and pri mary resistant strains have presented no sig nificant problem as yet

The occurrence of acute meningococcemia accompanied by vascular collapse (the Water house Friderichsen syndrome) calls for heroic measures including chemotherapy with both sulfadiazine (or Gantrisin) and penicillin hydrocortisone intravenously to relieve adrenal insufficiency and norepinephrine intrave

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The Gonococcus

INTRODUCTION

The gonococcus (*Neisseria gonorrhoeae*) is a gram negative coccus a species of the genus *Neisseria* (family *Neisseriaceae*) to which belong also the meningococcus and several non pathogenic inhabitants of mucous membrane It is the cause of a number of contagious infections of human columnar and transitional epithelium Hence urethritis cervicitis salpingitis and other complications of adults vulvovaginitis of children and ophthalmia of the newborn and adults are contagious inflammatory conditions caused in common by the gonococcus The commonest form of infection with this organism is gonorrhea known in the vernacular as clap or strain (Fig 2G)

HISTORY

While most authors suggest that gonorrhea is a disease of great antiquity there is no conclusive proof to support this contention Ancient Egyptian prescriptions early Chinese and Japanese writings Biblical and Vedic references are suggestive to some medical historians nonetheless all of the above are presented in such vague generalities that it is impossible to determine whether the writers had in mind a specific contagious urethritis or not It is admitted that the concept of the specificity of an infectious process was not known to the ancients nonetheless the clearest descriptions of the process do not suggest any inflammatory component or contagious character of the urethridites described Galen A D 130 first employed the term gonorrhea which may be translated as flow of seed As Vertue (1953) suggests Galen s own definition and description of gonorrhea is equivalent to what today is termed spermatorrhoea Support of this contention may be found in the contemporary writings of independent physicians such as Aretaeus the Cappadocian and Celsus of the first century A D From the beginning of historical medicine until the onset of the dark ages from Hippocrates to Galen and still later the medical profession showed no general recognition of venereal disease From the dawn then until the deepening twilight of the Dark Ages we have no evidence of medical knowledge of a contagious urethritis which would fit our current concept of gonorrhea With the raising of the curtain on human history at the end of the Dark Ages we find the first references in medical history to suggest that the disease we call gonorrhea had appeared in western civilization The medical bible of the day was still Galen and when a contagious urethritis began to appear a place for it consistent with Galen had to be found Quite logically the term gonorrhea was chosen to carry the burden Guillaume de Salicet of the 13th century presumably dealt with this disease and was aware of its venereal origin He attributed the affliction to impurities retained under the male

PREVENTION

The extreme degree of sensitivity to the action of the sulfonamide compounds has rendered chemoprophylaxis the most effective method of preventing clinical cases of meningococcal infection The efficacy of small doses of sulfadiazine in clearing the carrier state was demonstrated early in World War II and the knowledge was quickly applied to the institution of chemoprophylactic measures on a large scale with gratifying results While small doses resulted in the prompt disappearance of meningococci from the nasopharynx there was a general tendency for the organisms to reappear after an interval of a few days Larger prophylactic doses resulted in the disappearance of the organisms for appreciably longer periods of time The results of continuous prophylaxis (1 Gm of sulfadiazine per day over a period of months) may be illustrated by the experience of United States naval recruits in World War II Although the primary object of chemoprophylaxis was the reduction of infections due to *beta* hemolytic streptococci the virtual elimination of meningococcal meningitis in the recruits during an epidemic period was the most impressive result

In the light of these results it seems wise to give close contacts of frank cases of meningococcal disease brief courses of chemoprophylaxis with sulfadiazine or other soluble sulfonamide compound This is particularly important if (1) the close contacts are children and (2) exposure occurs during an epidemic period In the case of hypersensitivity to these compounds penicillin provides an adequate substitute Presumably the administration of a longer course of chemoprophylaxis to the members of a closed community would result in an appreciable period of freedom from these infections Such a program should not be initiated without due consideration for the potential complications arising from the emergence of drug resistant strains not only of meningococci but of other bacterial pathogens as well in the course of prolonged chemoprophylaxis (Cheever, 1945)

As in the case of other air borne infections characterized by a high carrier rate attempts to prevent the spread of the causative organism by isolation and environmental control measures have met with little success No vaccine is available

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an almost total prevention of serious complications of the disease in males Gonorrhea remains everywhere present and uncontrolled and is the largest and most challenging venereal disease problem in the western world

MORPHOLOGY

The gonococcus appears in the exudate of acute gonorrhea as a diplococcus with contiguous sides flattened or slightly concave resembling a pair of kidney beans and measuring from 0 6 to 1 0 micron in diameter In these exudates many polymorphonuclear leukocytes contain no ingested organisms while other phagocytes display from a few to uncountable numbers The gonococcus does not possess spores true capsules or flagella It is stained readily by aniline dyes and is gram negative Ordinary optical methods do not reveal any intracellular differentiation

Cells growing in laboratory media differ somewhat in appearance from those seen in pathologic material Cultures consist predominantly of single cells and small clumps but also frequently exhibit irregular staining giant forms Cellular morphology is progressively altered by contact with penicillin both in vitro and in vivo Smears of exudate taken during the first 4 or 5 hours of penicillin therapy show gonococcal forms that progressively enlarge and decrease in stainability until none can be detected Similar morphologic changes are observed during chloramphenicol therapy but not during sulfonamide treatment

CULTIVATION AND CHARACTERISTICS

Primary cultivation of the gonococcus on laboratory media is difficult not only because the organism is fastidious in its growth requirements but also because it is exceedingly susceptible to the toxic effect of a variety of substances commonly present in ordinary media The organism grows best under aerobic conditions at pH 7 2 to 7 6 at a temperature of 35 to 36 C Some strains do not grow satisfactorily at 37 5 C and in general growth stops below 30 C or above 38 5 C Most strains require an atmosphere containing from 2 to 10 per cent CO to initiate development Although gonococci grow well on the moist surface of solid media containing 1 2 to 1 5 per cent agar excessive moisture as produced by syneresis of the agar is undesirable especially for primary isolation because it favors other bacteria especially spreaders which readily overgrow the more slowly growing gonococcus Satisfactory growth is obtained on agar media consisting of meat infusion peptones glucose buffered with phosphate and enriched with plasma and hemoglobin or whole blood Increased yields can result from the addition to the media of yeast and liver concentrate which supply glutamine and cocarboxylase (shown by Lankford et al 1943 1946 to be essential for 10 to 15% of gonococcus strains) Glutathione has also been found by Gould (1944) to be an essential growth factor for certain strains As mentioned earlier many components of media exert an inhibiting effect on the growth of gonococci Thus certain amino acids occur in peptones in concentration sufficient to be somewhat toxic but this effect can be reduced markedly or abolished by heating the medium after addition of the blood (chocolate agar) Ley and Mueller (1946) have shown that agar also can exert an inhibiting effect on the growth of certain strains of gonococci and that the effect can be counteracted by the addition of charcoal or starch to the medium The inhibitory substance appears to be a fatty acid Since the gonococcus does not metabolize starch and since starch becomes ineffective after hydrolysis it appears that the beneficial effect of starch as well as of charcoal is due to its ability to adsorb the toxic agent serum appears to play a similar role

The bacterial flora of urethral and cervical secretions contain organisms that grow very much more rapidly on artificial media than does the gonococcus Such overgrowth prevents the detection of gonococcus colonies Media can be rendered more selective by the addition of certain dyes like Nile blue A or crystal violet in concentrations which are sufficient to inhibit many other bacterial species but allow growth of the gonococcus Advantage has been taken of all these facts in certain prepared commercial media such as Bacto GC medium base which are convenient for the isolation of gonococci from pathologic material

After 48 hours incubation the primary gonococcus colony appears translucent raised finely granular, slightly convex with lobate

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MORPHOLOGY

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margins It is usually mucoid and varies in size from punctiform to 5 mm in diameter depending on the medium and the crowding of the plate Although ordinary agar cultures die within 3 to 4 days unless transferred, agar slant cultures kept at 35° C remain viable for a considerable period of time if covered with sterile paraffin oil

Glucose is the only sugar fermented by the gonococcus with the production of acid but no gas Other members of the genus *Neisseria* may be encountered to the extent of 3 to 10 per cent in female secretions and 1 to 3 per cent in exudate from the male Fermentation reactions differentiate these species from the gonococcus The fact that the gonococcus produces an oxidase has been utilized as a diagnostic test When 1 per cent aqueous solution of dimethyl p phenylenediamine monohydrochloride is added to an agar growth the colonies turn pink then purple The reagent kills the organisms within a few minutes but does not modify their morphologic and staining characteristics The oxidase reaction is often negative in media containing 1 per cent glucose with the addition of neutralized reagent it can be rendered positive (Bucca Thayer and Schubert 1947) All members of the genus *Neisseria* as well as certain bacteria and yeasts occasionally found in the flora of the urethra and the cervix also can produce oxidase Nevertheless positive oxidase reaction coupled with typical colonial characteristics and the presence of diplococci resembling the gonococcus constitutes presumptive cultural evidence which should be confirmed by sugar fermentation Studies by Case (1957) show the oxidative enzyme to be associated with cell particles sensitive to heat and cyanide This enzyme is not cytochrome oxidase

Tonhazy and Pelczar (1953) have studied the oxidation of amino acids and compounds associated with the tricarboxylic acid cycle by the gonococcus Only the substrates from alpha keto glutarate through pyruvate were oxidized Histidine was deaminated Of the amino acids only D and L glutamic were oxidized rapidly They conclude that glutamate was oxidized mainly through the usual intermediates of the tricarboxylic acid cycle ending in acetate which the gonococcus can *not oxidize* Griffin and Reider (1957) have shown that various strains of gonococci will grow in the absence of CO atmosphere if yeast extract is added to the medium and that a combination of hypoxanthine uracil and oxaloacetate can replace the yeast extract Tryptophane and biotin were also found to be necessary in the metabolism of *N gonorrhoeae*

As mentioned under ordinary conditions the gonococcus dies rapidly in agar cultures It is killed quickly by drying, sunlight and ultraviolet light At 42 C death occurs within 5 to 15 hours in vitro (Carpenter et al, 1933) Moist heat at 55 C kills the gonococcus in a few minutes Phenol bichloride of mercury and silver compounds are very effective disinfectants The gonococcus is more soluble than the meningococcus in dilute NaOH (within 1 minute) whereas other *Neisseria* are practically insoluble in this reagent Although the gonococcus is susceptible to sulfonamides the range may vary from complete resistance of certain strains which can synthesize para aminobenzoic acid to susceptibility to drug concentration readily obtained in the blood Induced resistance in vitro to sulfonamides and streptomycin is acquired rapidly

The organism is extremely sensitive to penicillin complete inhibition of growth occurs with concentrations of 0 002 to 0 333 units per ml of medium (Love and Finland 1955) Penicillin X appears to be more effective in vitro and in vivo than the commercial product containing predominantly G penicillin Up to the present time no marked difference in mean penicillin susceptibility has been observed between strains isolated from cases of therapeutic failures and those isolated from patients successfully treated with the drug Using the technics of tissue culture intracellular gonococci may be protected against many times the minimal inhibitory concentration of penicillin chloramphenicol erythromycin novobiocin and the tetracyclines (Thayer et al 1957)

The gonococcus causes infection only in man numerous attempts to reproduce th disease in animals have failed Chemotherapeutic experiments have been carried out in embryonated eggs anterior chamber of the rabbit s eye and in the mouse by the intraperitoneal injection of gonococci suspended in mucin (Hill 1944)

Although different colony types of growth of gonococcus can be recognized Mahoney et al (1946) were unable to correlate colonial type and pathogenicity by injecting the organism into human volunteers

The toxicity of the gonococcus appears to be entirely due to an endotoxin The injection of heat killed gonococci results in toxemia and death similar to the injection of living cocci Nucleoproteins extracted from the organisms are almost as toxic for mice as are the organisms themselves Mice can be protected against its lethal effect by the injection of large doses of crude penicillin preparations So far other chemical components separated from the gonococcus have not proved to be of any practical significance in the development of tests u eful in the diagnosis of gonorrhea Failure to detect significant levels of antibodies by agglutination precipitation bactericidal complement fixation and allergy tests may be traced perhaps to the fact that the infection stimulates little antibody formation because of its local character However in controlled experiments volunteers displaying a positive gonococcus complement fixation test prior to inoculation did not develop disease with the same frequency as those recorded as having a negative test (Mahoney 1946)

CLINICAL COURSE

The usual incubation period of the natural infection is from 2 to 8 days and varied from 1 to 31 days in the experimental disease with a mean of from 3 to 5 days (Mahoney et al 1946) These experimental observations were closely paralleled by clinical observations in a large Prevention and Control Center (Garson 1953) and more recently in Finland (Haro and Patiala 1957)

The typical onset is sudden Usual symptoms consist of frequent urgent and painful urination and a profuse mucopurulent discharge The gonococcus is unable to penetrate stratified squamous epithelium however in the male the urethra with its stratified columnar epithelium is favorable for the pentration of the coccus (Harkness 1948) Penetration takes place through the intracellular spaces the organism being observed to reach the subepithelial connective tissue on the third or fourth day Polymorphonuclear leukocytes lymphocytes plasma and mast cells soon appear beneath the columnar epithelium being particularly numerous in the region of Littre s glands and ducts and the lacunae of Morgagni Large numbers of leukocytes carrying gonococci find their way from the acutely inflamed area into the lumen of the urethra The inflammatory response is caused by the elaboration of gonococcal endotoxin These with serum form the profuse yellow discharge characteristic of the disease The ducts of Littre s glands may become obstructed by leukocytes and desquamated epithelial cells resulting in the formation of retention cysts or abscesses Spread of the disease horizontally takes place by continuity from the subepithelial connective tissue and also directly into lymphatic vessels resulting in prostatitis and epididymitis Extension of pathology to subepithelial tissues and subsequent healing allows for contracture which in annular structures may cause narrowing and stricture In healing of the acute infection stratified squamous epithelium appears over the granulating surface of denuded areas which may compress and destroy any underlying columnar epithelium

In the female the cervical glands Skene s glands and Bartholin s glands are usual sites of primary infection The rectum is frequently the site of secondary infection Histologic pathology is similar to that described for the male urethra Involvement of cervical glands as for Littre s glands usually is confined to the ducts giving rise to a mucopurulent discharge of a degree ranging from very severe to mild

Progression to the fallopian tubes results in salpingitis usually bilateral There is a tendency for the tubes to close and form pyosalpinx and pelvic inflammatory disease Residual pathology in the tubal structures may require surgical correction after the inflammatory process has been brought under control In the glands of Bartholin unilateral inflammation is confined to the ducts and perigland ular tissue

Proctitis may occur more commonly noted in the female to the extent of 10 to 30 per cent Other complications occasionally encountered are conjunctivitis arthritis tenosynovitis meningitis endocarditis parotitis and sterility

N gonorrhoeae can be responsible for oph

thalmia neonatorum, an inflammation of the eye of the newborn resulting from infection during passage through the birth canal The condition which appears several days after birth is always serious, is frequently destructive to the ocular structures and before the use of silver nitrate prophylaxis, was credited with being responsible for 12 per cent of all blindness Today the recorded incidence is well under 0 5 per cent Because of the side effects of silver nitrate treatment the effectiveness of antibiotic prophylaxis (penicillin erythromycin and oxytetracycline) has been investigated These agents appear to be as effective as silver nitrate in protecting infant eyes against gonococcic infection

Gonococcal vulvovaginitis is an inflammation of the urogenital tract of prepubescent females and must be differentiated from that due to a variety of other infectious agents by bacteriologic means The disease is transmitted by intimate direct contact with infected adults and infrequently by contact with contaminated moist articles Cohn et al (1940) believe that epidemics rarely occur

DIAGNOSIS

Gram negative intracellular diplococci in the stained exudate from a suspected gonococcic infection strongly suggests the diagnosis of gonorrhea The intracellular position of the gonococcus is a common finding in acute gonorrhea but in very early or chronic infection the organisms may be found only extracellularly frequently as a single coccus In men a diagnosis based on the characteristic clinical symptoms usually can be confirmed by the finding of the intracellular gram negative diplococcus in pus cells in the urethral discharge and preferably by positive identification of *N gonorrhoeae* by culture technic including the determination of the sugar fermentation reactions Trauma or the introduction of mechanical and chemical irritants into the urethra may give rise to an inflammatory process having some of the characteristics of gonorrhea but it can be differentiated by negative bacteriologic findings Similarly a condition designated as nonspecific urethritis is encountered Instead of a burning sensation during urination as with gonorrhea an itching sensation of the urethral canal and pain referred to the glans occurs independent of urination The discharge is thinner gray to white in color and usually less profuse than gonorrhea It does not respond to penicillin therapy So far as is known no microbial agent other than *N gonorrhoeae* has the capacity to produce acute urethritis consistently in the human

Gonorrhea in women may vary in intensity from a circumscribed and almost symptomless inflammatory process to widespread disease involving the mucous membranes of the urinary and birth canal structures including the peritoneal covering of the adnexa Laboratory diagnosis is more difficult than in men Stained smears are of value in early infections when typical intracellular organisms may be found in material from the urethra or the cervix As the age of the infection advances the value of the spread finding decreases while that of the culture method increases The relative reliability of the two methods has been studied in incarcerated prostitutes by Van Slyke et al (1942) Specimen material was collected from each of 140 patients with clinical gonorrhea and positive culture findings Stained preparations were prepared from each patient in triplicate and the slides were examined by 3 experienced microscopists who reported as positive only 88 47 and 40 of the specimens respectively Although the culture method is more reliable it has also distinct limitations as practiced at the present time In 3 laboratories employing identical technical methods and testing samples of identical material complete agreement could be obtained in only 75 per cent of patients known to be infected

It should be pointed out in this connection that in chronic untreated gonorrhea culture findings may spontaneously reverse from positive to negative without any accompanying change in the clinical status of the infection Furthermore sporadic positive cultures may appear after a relatively long series of negative cultures (Mahoney et al 1942)

Complement fixation tests (gonoreaction) at present are not sufficiently reliable to be used in diagnosis Employing the Price and Kolmer methods with single and multiple strain antigens Van Slyke et al (1942) observed many instances of clinical gonorrhea with positive cultures but negative complement fixation findings Nørgaard (1956) reports that the gonoreaction was negative in

85 per cent of males and 70 per cent of females with uncomplicated gonorrhea

Difficulties inherent in the isolation of the gonococcus are increased when the clinician fails to exercise care in securing suitable exudates for cultivation This is especially true of specimens obtained from women since the bacteria present in the flora of cervical exudate may overgrow the gonococcus Inoculations from gonorrheal exudate were found by Schubert Bucca and Thayer (1947) to be most successful when made in the clinic directly on the agar surface and incubated immediately When this is not possible the swab containing the exudate should be placed in a small amount of broth and taken to the laboratory

The demonstration of gonococci in cervical exudate is said to be most favorable during the postmenstrual phase However other workers prefer the premenstrual phase and some even choose the menstrual period Koch (1947) studied a small series of patients and concluded that positive cultures are associated with the estrogenic phase when the pH of the cervical mucus is 6 8 or above while negative cultures are associated with the luteal phase of the cycle when the mucus is most acid Putkonen (1950) studied this problem in 343 patients hospitalized for gonorrheal cervicitis in which menstruation was known in relation to 1 119 examinations He found no significant variation of positive cultures in relation to the day of the menstrual cycle

Methods for the transportation of suspected secretions from the patient to a distant laboratory for cultural diagnosis have not been very successful An ideal transport medium must preserve the viability of the gonococcus and at the same time prevent overgrowth of contaminating bacteria Inhibitory substances such as crystal violet Nile blue A thallium acetate and tyrothricin when added to enriched media suitable for gonococcus preservation and growth are not sufficiently selective for contaminating bacteria resulting in death of certain sensitive gonococci Stuart (1954) uses a buffered nonnutrient agar medium to which thioglycollate is added to prevent oxidation In theory viable gonococci are preserved and contaminants fail to grow for lack of nutrients He finds 95 per cent recovery of positive cultures when plating takes place under 12 hours and 83 per cent after 24 hours After 48 hours delayed plating recovers 77 per cent

Specimens of blood synovial fluid and spinal fluid are cultivated best in shallow layers of ascitic fluid broth in an atmosphere containing from 2 to 10 per cent CO where the organisms grow as a mucoid sediment on the bottom of the flask Urine preferably the first morning specimen should be centrifuged and the sediment inoculated on agar medium Taggart (1955) has proposed a rapid screening procedure for the detection of gonorrhea The technic consists of macroscopic inspection followed by microscopic examination of the presumptively positive urine sediments for the presence of *N gonorrhoeae* Tested in an out patient service of a general hospital where the patients were not appearing primarily for venereal disease gonorrhea was discovered in 15 per cent of the males and 6 8 per cent of the females In an extension of this technic a field trial was carried out among the Ute and the Navajo tribes by the Indian Health Service of the U S Public Health Service The procedure was found to be of limited value in screening large populations under such field conditions Prostatic fluid may be directly inoculated on agar or centrifuged in a small amount of urine and the sediment inoculated on agar

For more detailed information concerning the cultural method of isolating and identifying *N gonorrhoeae* the reader is referred to the following sources Carpenter et al (1949) Thayer Schubert and Bucca (1947) and Public Health Service Publication No 499 (1956)

In general because of the characteristic clinical symptoms presumptive cultural evidence of gonococcal infection (unconfirmed by sugar fermentation) is more acceptable in the diagnosis of gonorrhea in the male than in the female In the female where clinical evidence is of little value a negative smear report coupled with presumptive cultural evidence may lead to as many as 16 per cent false positive diagnoses For example Thayer (1943) obtained oxidase positive colonies of various micro organisms from 36 per cent of 548 normal uninfected women In 3 4 per cent the cultures were saprophytic *Neisseria* and in 12 6 per cent the organisms were morphologically and tinctorially so similar to the gon

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While there is no evidence of acquired absolute or increasing resistance of N gonorrhoeae to penicillin the work of a number of investigators from 1944 to date indicates that the range of sensitivity of the organism to penicillin varies from 0 007 to 0 333 units per ml The relatively resistant strains of the organism in cases of presumed treatment failure on routine dosage schedules will respond satisfactorily after laboratory determination of sensitivity, to a dose schedule arranged upward appropriately to meet needs

In the past decade approximately 17 antibiotics other than penicillin have been discovered and made available commercially to the physician Nine of these 17 have been shown to have an effectiveness comparable with penicillin when used in varying dosage for clinical gonorrhea None of the newer antibiotics is a serious competitor of penicillin in the treatment of gonorrhea when one considers such factors as mode of administration dose toxicity side effects and cost Although these agents offer no advantage over penicillin they should be considered in the treatment of gonorrhea in patients known to be sensitive to penicillin

A brief comment at this point concerning penicillin reactions would seem to be indicated Although such reactions have been reported as occurring in up to 6 per cent of patients receiving penicillin the largest study in the United States carried out by C A Smith et al (1954) on penicillin reactions occurring in venereal disease clinic populations indicated that only 114 reactions of all types were reported in 19 288 patients observed In this group there were only 4 anaphylactoid penicillin reactions The total reaction rate of less than 1 per cent in this population group is encouraging however the prospect of having to handle the sudden emergency of penicillin anaphylaxis which can terminate fatally in a matter of minutes should hasten the reader to other sources in the literature concerning the proper handling of these situations No physician or clinic should undertake the treatment of patients with penicillin without having at hand the equipment the drugs and the professional competence to handle such an event

Adequate criteria of cure in men consists of complete freedom from clinical evidence of the disease Examination for evidence of residual gonococcic infection may be done about 7 days after the completion of therapy The test of cure if done at all should consist of cultures The material for culture should be obtained from urine sediments Expression of the urethral glands by gentle massage of the canal over a sound and prostatic massage has been employed in the past although instrumentation as an aid in determining the presence of gonococci is not necessary and is not advised In general a test of cure is considered to be unnecessary in males in the absence of signs or symptoms of gonorrhea however epidemiologic evidence of the infection of sexual partners in the absence of signs and symptoms of the disease would necessitate further bacteriologic studies The tendency of the infection to produce asymptomatic carrier states is not great and most if not all patients harboring the gonococcus will display soon or late clinical evidence of infection In women repeated cultures of carefully obtained and selected material from the cervix Skene s glands and Bartholin s glands offer the only available means for detecting residual infection At least one culture should be obtained from these 3 sites from 7 to 10 days after treatment If prolonged acting penicillin has been utilized in treatment it is of course necessary that a proper amount of penicillinase be incorporated in the culture medium As a precautionary measure in both men and women unprotected sexual exposure should be interdicted until the criteria of cure have been satisfied Relapse if it occurs will be seen most commonly in the first week after treatment Occasionally it is found that what appears to be a relapse or treatment failure is a reinfection contracted from a regular sex partner who has not been given treatment or is undiscovered for lack of proper epidemiologic studies

An exceptional circumstance is presented when gonorrhea and syphilis or other venereal diseases are contracted concurrently That this is not such a rare occurrence is attested to by the findings of the Venereal Disease Clinic in Vancouver British Columbia which showed that 3 per cent of the patients attending primary clinics simultaneously had acquired

ococcus that sugar fermentation tests were necessary to rule out this organism

TREATMENT

The advent of the era of chemotherapy subsequent to 1935 saw the rapid decline of the treatment of this disease by such practices as the local application of antiseptic and astringent solutions by means of injection irrigations and installations and of attempts to alkalinize the urine All of these methods are of questionable efficacy and may have added to the complications of gonorrhea noted in those times The use of sera vaccines and culture filtrates has also proved to be of very limited if any value Artificial hyperpyrexia based upon the marked heat lability of gono cocci has been practiced but has certain in herent dangers and technical difficulties

When the sulfonamide compounds and sub sequently penicillin became available the therapy of gonorrhea passed from the province of urology and to a lesser extent of gynecol ogy, to that of chemotherapy largely in the hands of the general practitioner

Although the recent recommended practice of the employment of 600 000 units of a re pository penicillin in a single intramuscular injection for the routine treatment of uncom plicated gonorrhea has given excellent results in males Preston and Dunsworth (1957) Hookings and Graves (1956 57) and others have demonstrated at least a 13 to 15 per cent failure rate on females in clinic populations In general it has been alleged that as much as 2 to 5 per cent of males and 10 to 20 per cent of females failed to obtain cure by the single intramuscular injection of 600 000 units of repository penicillin Such observations have taken into consideration the possibility of reinfection in such patients The reason for such treatment failures in the face of the rather remarkable susceptibility of the gono coccus to penicillin has been explored by Thayer et al (1956 57) Other than purely technical failures in the use of the drug recent work suggests at least two reasons for treat ment failures The first concerns itself with the relative resistance of certain strains of N *gonorrhoeae* to penicillin in relation to the blood levels in time obtained when using re pository penicillin The second, unsubstanti ated by clinical research, is the observation in tissue culture studies that the gonococcus gains access to the intracellular milieu of cer tain body cells remains viable intracellularly and is protected from extracellular antibiotic It is hypothesized that in time with the dis solution of the host cell, viable gonococci are available for the auto infection of the host thereby allowing for treatment failure While the former situation probably represents the main cause for the bulk of the problem if indeed the second set of circumstances actually exists in human beings nonetheless such ob servations have stimulated the U S Public Health Service and various State Health De partments to recommend higher doses of the longer acting repository penicillins in the treatment of gonorrhea The recommended treatment of choice currently utilizes either procaine penicillin G in oil with 2 per cent aluminum monostearate or benzathine peni cillin G in the following doses

Uncomplicated gonorrhea in males—either type of penicillin preparation 600 000 to 1 200 000 units in 1 intramuscular injection

Uncomplicated gonorrhea in females—either penicillin product 1 800 000 units intramus cularly or 600 000 units of penicillin alumi num monostearate plus benzathine penicillin G, 1 200 000 units intramuscularly in one or two injections at one visit

Gonorrhea with complications—aqueous penicillin G 600 000 to 1 800 000 units per day at intervals of 2 to 4 hours or equivalent amounts of repository penicillin until signs and symptoms have subsided

Retreatment usually is indicated if upon the exclusion of the likelihood of reinfection the discharge in uncomplicated gonorrhea per sists for 3 days or more after initial treatment and smear or culture is still positive In gen eral the retreatment dose consists of a dou bling of the original treatment schedule at a single session or equally divided injections of the increased dose over a period of 3 to 5 days until the total dosage is achieved and signs and symptoms have subsided

The need for local therapy prostatic mas sage or artificial hyperpyrexia is seldom if ever encountered So-called prophylactic or epidemiologic treatment should be given to all sexual contacts of a positively diagnosed case of gonorrhea The recommended treatment for

basis Spontaneous recovery of the anterior urethritis in men is usual eventually in the absence of excessive sexual activity professional trespass or reinfection Ricord's statement over a century ago is at least still applicable to such recovery in women "We know when gonorrhea begins but God alone knows when it will end" Acquired immunity has not been demonstrated and one attack does not protect against subsequent infection It has been demonstrated that males vary greatly in their resistance to experimental infection Mahoney et al (1946) inoculated a large number of male volunteers by installation of massive doses of cultures into the urethral canal In a total of 245 experimental exposures typical clinical disease with confirmatory laboratory findings was produced in only 83 or 33 8 per cent In the remainder a train of irritative symptoms of varying severity that persisted for periods ranging from several hours to 2 days was followed by a return to normal The relatively low incidence of the infection among prostitutes may possibly be the expression of such natural resistance although it is known that the well initiated among this group practice a wide variety of empiric prophylactic technics in the process of "scrubbing the decks" aimed at professional self preservation In culture studies carried out over several years Van Slyke et al (1942) found approximately 20 per cent of incarcerated prostitutes and other sex offenders to be infected

National morbidity statistics compiled by the U S Public Health Service over the past decade indicate a decline in reported cases of gonorrhea From fiscal years 1947 to 1956 there was a 40 per cent decrease in reported cases of gonorrhea and a 47 per cent decrease in the rate while infectious lesion cases of syphilis declined 94 per cent during the period in this country Any optimism concerning this favorable trend in morbidity should be tempered by the fact that during this period simplified antibiotic treatment was developed and more and more cases were seen by private physicians whose reporting of gonorrhea dropped to an all time low during this same period of time Furthermore there has been an almost inconsequential decrease in the rate and cases reported during the 5 years from 1952 to 1956 Again it must be remembered that these morbidity statistics represent only a portion of the actual cases occurring During the fiscal year 1956 the State Health Departments reported a total of 233,333 cases of gonorrhea exclusive of cases occurring in the Armed Forces It has been estimated that the actual incidence of gonorrhea at that time was between 5 and 10 times the number of cases reported

PROPHYLAXIS

Mechanical prophylaxis by means of a condom offers the only type of protection upon which any reasonable reliance can be placed In the male transmission of the disease is effected by the contamination of the very distal portion of the urethral mucosa On hypothetical grounds any chemical agent capable of destroying *N gonorrhoeae* while the organisms occupy a vulnerable position on the surface of the mucous membrane should serve as an effective prophylactic However there is no acceptable evidence that the commercial chemical prophylactic preparations or those used in military organizations have any appreciable degree of effectiveness In women mechanical cleansing with soap and water or with mild antiseptic solution offers a theoretical but unconfirmed mode of protection The prophylactic use of penicillin has been explored by the Armed Forces in areas where high incidence of gonorrhea is encountered The utilization of a single oral benzathine penicillin G tablet (250 000) units is employed in the following fashions either upon embarking for short term leave or upon return from leave 1 penicillin tablet is given to all personnel or upon reporting for prophylaxis after exposure The employment of such a technic on all personnel while presumably necessary under certain circumstances has been criticized from the standpoint of initiating penicillin sensitivity in a certain proportion of the personnel and the rather large cost of such a program per gonorrhea case prevented In a well disciplined group it would appear that the best utilization of this technic would be upon personnel reporting after exposure In any event this measure is considerably more effective in the prevention of gonorrhea than the chemical prophylaxis formerly utilized by the Armed Forces All contacts of patients with gonorrhea should be identified

syphilis and gonorrhea In these instances, the gonococcal infection because of its short incubation period becomes evident while the syphilitic infection is still in the preclinical or incubation stage Penicillin therapy directed toward the cure of gonorrhea, if in the amount and the type of preparation suggested previously will allow for the aborting of an oncoming syphilitic infection in practically all instances The occurrence of chills or fever (Herxheimer reaction) accompanying penicillin therapy of gonorrhea is strong presumptive evidence of coexistence of syphilis as shown by Fromer Cutler and Levitan (1946) Hence all patients who are to receive penicillin treatment for gonorrhea should have a serologic test for syphilis *prior to or at the* time of treatment and monthly for 4 months after the completion of treatment The same precautions should be observed in patients receiving prophylactic treatment and treatment with other antibiotics

EPIDEMIOLOGY

Since the epidemiology of gonorrhea concerns itself with the various factors and conditions which determine the occurrence the frequency and the distribution of the disease and its sequelae among the population it is necessary for an understanding of the epidemiology of this disease to have a fairly reliable body of knowledge concerning the incidence the prevalence and the natural course and history of the disease Reliable information on incidence and prevalence of the disease depends upon faithful reporting of diagnosed cases As previously indicated it is extremely difficult to diagnose some cases of gonorrhea particularly in the female This fact, plus the inadequacy of reporting cases makes it practically impossible to accumulate any reliable data on the prevalence of this disease As a consequence figures concerning the incidence represent only a portion of the true picture Although the situation concerning the body of knowledge referable to the natural history and course of this disease is considerably better than information available on incidence and prevalence nonetheless there are yet tantalizing unknowns and inconsistencies in our knowledge which further complicate the situation in relation to the epidemiology of this disease The long known relationship of the social and behavioral factors of promiscuity sexual freedom and irresponsibility to the continued presence of this disease in society is well established All manifestations of gonococcal infection are maintained in a population by the continued presence of the venereal form which in turn is maintained by indiscriminant sexual behavior

The distribution and the occurrence of gonorrhea is world wide Available data would suggest that it is a common disease among persons of lower socioeconomic status however it is not restricted to this group It affects both sexes and practically all ages but particularly the age groups of greatest sexual activity In the United States the highest *incidence is found in the age group 20 to 24* years Many states indicate that 25 per cent of the gonorrhea reported occurs among teen agers Studies of the U S Public Health Service have indicated that 50 per cent and more of the individuals involved in epidemics of gonorrhea are also in the teen age group The source of infection is exudate from mucous membranes of infected persons and man is the only known reservoir Carriers characterized by the harboring of virulent organisms in the structures of the urogenital tract who are otherwise asymptomatic have been reported recently from England and the United States Fortunately the percentage of such individuals is relatively small but significant in terms of the control of the disease The mode of transmission is almost wholly by sexual intercourse with the exception of the ophthalmic infection acquired by the newborn during passage through an infected birth canal Intimate contact on the proper type of mucous membrane is absolutely essential for the transmission of this disease and the role of intermediary objects in transmission may be considered as inconsequential As indicated elsewhere the incubation period has wide variation while the period of communicability may be from months to years unless interrupted by adequate therapy which ends communicability within hours or days Susceptibility is general and instances of racial immunity have not been recognized The popular belief that an infection contracted from a member of a certain race or in a certain geographic area is more severe or more resistant to treatment than others has no factual

gonorrhea would decline similarly to *syphilis* in the face of penicillin therapy did not properly consider that the syphilis control program operated with other effective epidemiologic technics for which there was no operational counterpart in the attempts to control gonorrhea As a consequence the hoped for decline in the prevalence and the incidence of gonorrhea similar to that noted in lesion cases of syphilis (94% reduction in past decade) has not occurred

Becoming aware of the continued limitations of gonorrhea control programs the U S Public Health Service in 1952 with the co operation of State Health Departments and selected large cities in the United States initiated the first major attempt to incorporate known epidemiologic information into a methodology for the control of gonorrhea The technic was called speed zone epidemiology Speed zone epidemiology (peppy epi) is an attempt to apply specialized technics in gonorrhea case finding interviewing and investigation of contacts as well as patient education which will exploit the characteristics of this disease Mahoney et al (1946) had demonstrated that the incubation period of experimentally acquired gonorrhea in males has a 31-day range but that 85 per cent of the infections produced clinical symptoms within 6 days Presumably then the bulk of the case finding problem could be narrowed down in at least 85 per cent of all cases to the epidemiologic investigation of sex contacts of gonorrhea patients for the period beginning 6 to 7 days prior to the onset of clinical symptoms and ending at the time of appearance at the clinic If valid information were obtained at interview of the patient with gonorrhea the source case and the pread or contact cases would be identified and hopefully located and brought in for treatment thereby breaking the chain of gonorrheal infection surrounding the particular patient Since at that time penicillin in aluminum monostearate was the longest acting penicillin available and presumably protected the patient from reinfection for at least 72 hours and since reinfection from the same sources without such protection was known to be common and since developed interviewing technics had been proved to be highly effective in identifying contacts investigation of the patient s contacts to be effective in the control of gonorrhea had to be accomplished within the treated patient s penicillin protective period or 72 hours Rapid or *speedy investigation is therefore fundamental* to the operation of such a program whose aim is to break the chains of infection and protect the patient from reinfection by his sex contacts The immediate problem was the development of procedures to facilitate such rapid investigation The most commonly employed technics to bring sex contacts to clinic observation within the 72 hour period were (1) an immediate telegram to the contact requesting immediate appearance at the health department (2) an immediate telephone call to the contact (3) the immediate assignment of the contact not having an adequate address telephone number or responding to these technics to a venereal disease investigator and (4) having the informant bring in his contacts within 24 hours of the informant s treatment

Only male patients with diagnosed gonorrhea were interviewed for sex contacts Most well run large clinics had observed that the stimulus supplied to the patient by gonococcal urethritis was sufficient to attain excellent volunteer reporting by males As the females in most all instances were completely unaware or belatedly aware of their gonococcal involvement female volunteering could not be depended upon in gonorrhea control Hence adequate case finding among females depended upon male volunteering and co-operation in the identification of sex contacts The next essential in such a program other than the treatment of the original case is the immediate routine treatment of all sexual contacts brought to the observation of the clinic whether they are diagnosed upon observation as gonorrhea or not This latter feature is further essential as the limitations of diagnosis of gonorrhea in the female have been well established and if treatment is given only to those diagnosed upon observation as much gonorrhea as is diagno ed and treated will be returned untreated to the population to further the spread of this di ease

Utilizing this technic in a large mid Southern city over a 1 year period it was possible to obtain an index of 1 7 sex contacts per patient interviewed bring approximately 85 per cent of the sex contacts to clinic observation and treatment within 72 hours with 50 to 75 per cent of the contacts being treated within 24 hours it allowed for a reduction in morbidity over the previous year of approximately 1 500 cases of gonorrhea That all such speed zone epidemiology programs were equally effective or that a marked reduction in morbidity was continued yearly is far from the fact however the important feature of

located and treated as soon as possible Of course such contacts should be examined to *determine their diagnostic status* but regard less of whether the examination indicates the presence of gonorrhea or not all such con tacts should be treated on epidemiologic or prophylactic grounds because of delays and difficulties in diagnosis particularly in females and the practical fact that sexual exposure has been demonstrated to continue despite advice to the contrary under such circum stances Every effort should be made to include all sexual contacts of the 6 to 10 days prior to the onset of the clinical infection as well as those contacts occurring from the time of onset until the *patient is first seen*

CONTROL MEASURES

In man s colorful past the attempt to con trol disease in his environment has varied from genocide and the weird muttered incantations and rites of the witch doctor to the attempt to drive away noxious vapors ' by the burn ing of bonfires and the shooting of cannons *In more recent times the enlightened have* built a structure for the control of communi cable diseases based upon 4 major principles namely quarantine immunization eradication of the intermediate host and specific treat ment Throughout the course of these events mankind has been aided indirectly by natural mechanisms which in many instances allow for naturally acquired immunity upon recovery *from disease or removal of a possible source* of infection by death of the individual When we attempt to apply the effective principles enumerated above to gonorrhea we become more fully aware of the difficulties in the con trol of this disease The success of the quaran tine of cases and contacts depends in large measure upon the capacity and the resources available to diagnose cases and find contacts The admitted deficiencies in our ability to diagnose this disease particularly among females and the staggering job of attempting to find and process properly and handle per haps 2,000 000 of our population is both im practical as well as *politically unfeasible in* our democracy We have no means of artificial immunization for this disease and to worsen the situation no effective natural immunity is acquired by having the disease As man is the only known reservoir of gonorrhea and there is no intermediate host which can be eradicated this principle does not apply If properly utilized, a specific treatment is avail able however there never has been an in stance in which specific therapy alone, given to diagnosed cases without aid of one or more of the other principles of control, ever has established or maintained control of a social disease of this nature

Is it any wonder then that the methods which have been used in the past for the con trol of gonorrhea, consisting largely of the diagnosis and treatment of the infected indi vidual have not been satisfactory? For gon orrhea specifically failure to control may be *attributed to the inadequacy of case reporting* extreme difficulty of diagnosis (especially in women) the fact that one infection does not produce an immune response sufficient to pro tect the patient against reinfection the lack up to recent times of a treatment suitable for mass therapy and the fact that specific therapy even if properly applied allows for the return to the population of a susceptible *individual within hours to days after treat* ment This latter factor of converting infected cases of gonorrhea in a matter of hours after treatment to susceptibles and returning them to the very venereal disease milieu from which they came with the not uncommon result of having individuals return presently as reinfec tions more than offsets the advantage of pres ent treatment It had been assumed that the *general use of penicillin therapy* would both reduce the period of infectiousness and de crease opportunities for transmission That these assumptions are correct there can be no argument but the further assumption that these factors alone would greatly *modify the* epidemiology of gonorrhea and bring about a marked decline in the prevalence of the dis ease is not valid It is an old saw that in solving a problem many times we make new problems In this instance the solution of one facet of the problem led to and was largely offset by the increased opportunity for rein fection In a short incubation period disease *such as gonorrhea, an increase in the* number of reinfections in a given period of time will greatly swell the morbidity statistics and tend to maintain the disease in society Aside from not taking into consideration the likelihood of this latter development those who *felt that*

will continue to be ubiquitous and common place in our society In the above regard and to place in proper perspective the work of medicine and public health in gonorrhea control the following statement by Dr C A Smith formerly Chief of the Venereal Disease Program U S Public Health Service is offered for thought

Venereal disease is unique among the communicable infections in that it is not wholly a clinical problem Its roots lie in the socially unacceptable environment in social maladjustment The venereal disease control program deals with the dilemma that is apparent that is the presence of a venereal disease Our services are specific we treat the infected individual and try to give him enough information so that he will not become reinfected or if he does that he will know what to do about it However we only skirt the edges when we look at the clinical aspects of the disease and do not deal with the deeper social causes or remote origin of the person s present difficulties

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these programs is the demonstration of the possibility of rapid investigation, their undoubted partial effectiveness in the reduction of gonorrhea in the community and the stimulus they afford to pioneer new and better epidemiologic technics for more practical and economic control of this disease

Excellent results of the use of this technic in Canada by Anderson and Nelson (1954) and Nelson (1957) have been reported A most worthwhile and interesting by product of speed zone epidemiology programs in gonorrhea has been the rather dramatic reduction in syphilis in the same population groups with no other added effort being expended other than the usual technics which were in vogue prior to the addition of the gonorrhea program Presumably the significant reduction in *syphilis* observed under these circumstances is due to the aborting of incubation or preclinical syphilis in the population that is rapidly sought out and treated liberally with penicillin on a fairly continuous basis This *appears to be a logical explanation* as it has been observed that the bulk of infectious syphilis occurs in the same socioeconomic and racial group of the population having the largest proportion of gonorrhea

As previously indicated speed zone epidemiology as a technic for gonorrhea control is hardly the panacea This is understandable when one considers that even in the best such programs one starts by allowing at least 15 per cent of the gonorrhea contacts to escape investigation As indicated above 15 per cent of the contacts named within the jurisdiction of the health department are not located and brought to treatment When one adds to these shortcomings the occasional failure at interview to obtain any contacts from the patient with gonorrhea contacts listed outside the jurisdiction of the health agency the misinformation given by the informant to cover up his real contacts as well as the undetermined number of unknown cases of gonorrhea in the population at any one time through the failure of reporting cases or the failure of the infected individual to seek ethical medical aid it is even more understandable why the technics developed to date have not been as effective as some of the more enthusiastic proponents had hoped The presence in the population of such a number of cases of a short term incubation period disease such as gonorrhea escaping *the epidemiologic net* would be sufficient to act as a continuing reservoir of infection in the community

The recent observations of Hookings (1956 57) on the utilization of combined penicillin aluminum monostearate and benzathine penicillin G in the gonorrhea speed zone project in Memphis, Tenn represents perhaps the first breakthrough in increasing the effectiveness of such control programs since their initiation in 1952 On January 1 1956 the Memphis clinic began to treat female contacts and patients with 600 000 units of penicillin aluminum monostearate plus 1 200 000 units of benzathine penicillin G At the same time the records of all female contacts were tagged so that any repeat visit to the clinic would be readily recognized By the end of 60 days the repeat rate which had been 15 per cent in 1955 had dropped to 1 7 per cent A decline was seen in the weekly male attendance at the clinic when compared with the previous 2 year period On April 1 1956 males as well as females began to receive the combined treatment Since that time there has been an increasingly marked decline in male as well as female attendance at the clinic *Careful scrutiny* of all other factors which could account for such a decline were made and no other factor than the change in the treatment schedules utilized in the speed zone program could be demonstrated Continuation of this approach for the remainder of 1956 allowed for a decrease in morbidity of approximately 1 100 cases over the previous year

Health department practice calls for

1 The stimulation of efficient case reporting in order that health organizations be informed as to the trends of prevalence incidence and distribution of the disease

2 The providing of adequate facilities for interviewing and prophylactic or diagnostic treatment

3 The rapid investigation of all sources of infection with the bringing to clinic observation during the period of penicillin protection from reinfection of the patient of individuals *sexually exposed to a known infected person*

4 The conduct of such informational educational and health promotional efforts as the situation in any given community may require both as regards the disease itself and the broader lines of social hygiene sex education and family living

5 The stimulation of and co operation with other resources and social agencies within the community whose programs can be effectively brought to bear upon the problem

Until the above practices are utilized widely and faithfully we may expect that gonorrhea

chronic course with intervals of clinical quiescence and relapse (3) lesions of the skin and the bones (4) presence of the same type of serum antibodies (5) prompt response to penicillin therapy and (6) occurrence predominantly among individuals living under poor hygienic conditions

The *Treponema* are slender spiral organisms which are readily distinguishable on morphologic ground from the *Borrelia* and the *Leptospira* the morphologic characteristics will be described under *Treponema pallidum* which is the type species of this genera A good working classification is the following although this arrangement has no standing in more formal taxonomic circles

1 Human Pathogens Primarily

A *T pallidum* The causative agent of syphilis

B *T pertenue* The causative agent of yaws

C *T carateum* The causative agent of pinta

D Treponemes that cause other human treponematoses such as bejel and other non venereal syndromes

2 Animal Pathogens Primarily

T cuniculi The causative agent of rabbit syphilis

3 Human or Animal Saprophytes Primarily

Included in this group are spirochetes showing the general morphology of treponemes occurring in the oral cavity about the gum margins and about the anus and in fecal material The designations *T microdentium* and *T macrodentium* depending on their size have been applied to these organisms In contradistinction to those in categories 1 and 2 above these organisms can be cultivated on artificial media and a number of strains so cultivated have been given distinguishing names such as the Reiter Kroo Noguchi Kazan and Nichols strains

Since syphilis and its causative organism *T pallidum* have been the most extensively studied the fundamental biology of this disease will be described at some length In considering the other treponemal syndromes particular attention will be directed to pointing out either differences between them and syphilis or limitations in our knowledge of that particular entity Bibliographic details for most of the investigators cited will be found in the second edition of *Bacterial and Mycotic Infections of Man* by Rene J Dubos 1952 or in *Biology of the Treponematoses* by Thomas B Turner and David H Hollander 1957

The question of the biologic relationship within the *Treponema* group of spirochetes has been studied by Turner and his associates (Turner and Hollander 1957) among others and will be summarized briefly in the final section of Treponema and the Treponematoses

TREPONEMA PALLIDUM AND SYPHILIS

HISTORY

Syphilis became epidemic in western Europe in 1492 and the immediately succeeding years It is generally believed to have been acquired by Columbus crew in the West Indies and brought by them to Europe but only meager evidence supports this hypothesis It appears more likely that syphilis and related treponematoses were endemic in Africa and Europe centuries before and reached epidemic proportions during the mass movements of armies and populations at the end of the 15th and the beginning of the 16th centuries The extraordinary virulence of the disease at that time appears clearly in some of the early descriptions but by the middle of the 16th century syphilis had become milder It is not known whether the present relatively mild course of the disease in its early stages reflects a change in the organism or a gradual development of resistance in the human host The causative organism was identified in 1905 when Schaudinn and Hoffman demonstrated its presence in the primary lesion and in the adjacent lymph glands of syphilitic patients Noguchi and Moore subsequently found the organisms in the cerebral cortex of patients dying with general paresis thus they proved that syndrome to be as had long been suspected a late manifestation of syphilitic infection

CHARACTERISTICS OF THE TREPONEME

Morphology *Treponema pallidum* is a fine spiral organism measuring 5 to 20 μ in length and about 0 1 to 0 2 μ in thickness it is difficult to see in the ordinary light microscope

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The Spirochetes

INTRODUCTION

The order *Spirochaetales* includes diverse groups of spiral and actively motile micro organisms the majority of which divide by transverse fission Classification of the Spiro chetes has been principally on morphologic grounds and within each subgroup there are both pathogenic and nonpathogenic species There has been a tendency to attribute rela tionships among these diverse groups merely because of their spiral form when in fact other and perhaps more important biologic relation ships are lacking As is the case in many other groups of bacteria the particular biologic properties that confer to one species and not to another the ability to produce disease in man or animals are often obscure however it is probable that the active motility of these organisms plays a significant role in their disease producing proclivities The spirochetes historically and still today account for a sub stantial segment of human disease

The spirochaetales have been divided into 2 families and 6 genera The family *Spiro chaetaceae* comprises 3 genera nonpathogenic for man *Spirochaeta Saprospira* and *Cristi spira* All are large flexible and undulating spiral organisms measuring from 30 to 500 μ in length the genera are distinguished by cer tain morphologic characteristics *Spirochaeta* are found principally in sewage and contami nated water *Saprospira* in mud and sand and *Cristispira* in oysters and other molluscs All grow best at approximately 20° C

The *Treponemataceae* comprises 3 genera each of which contains species pathogenic for man the *Treponema* to which belong the causative organisms of syphilis yaws pinta and the other treponematoses the *Borrelia* which comprises the large group of relapsing fever spirochetes and the *Leptospira* to which belong various species causing lepto spirosis It is well to keep these 3 large groups of pathogenic spirochetes clearly separated for they have little in common except a spiral form which however is distinctive for each genus It should be noted too that each group contains species that are pathogenic for man others that are pathogenic for animals but not for man and still others that appear to be only saprophytic for man or animals In general, their pathogenicity is not indicated by any morphologic characteristic and usually can be determined only by animal inoculation

The foregoing classification while useful and biologically justifiable has not been adopted universally and much confusion has been caused by the use of the term ' spiro chete and the generic classification *Spiro chaeta* for any spiral organism For example the terms *Spirochaeta pallida* and *Treponema pallidum* are used almost interchangeably *Borrelia recurrentis* is frequently referred to as *Spirochaeta recurrentis* and even at times as *Treponema recurrentis* or *Spironema re currentis*

TREPONEMA AND THE TREPONEMATOSES

The treponematoses are a group of clinical and epidemiologic syndromes which have many features in common including (1) eti ologic agent belonging to the *Treponema* group of spirochetes (2) subacute and

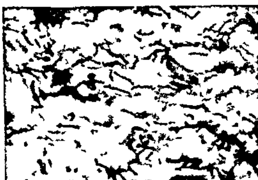


Fig 52 (*Top*) Congenital syphilis of the lung *Treponema pallidum* demonstrated by Levaditi s method (Smith L W and Gault E S 1942 Essentials of Pathology ed 2 New York Appleton Fig 121)

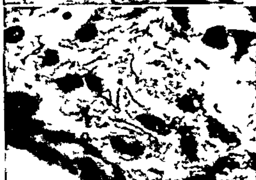


Fig 53 (*Bottom*) *T pertenue* of the skin secondary yaws Oil immersion photomicrograph stained by Levaditi s method The organisms appear as irregularly twisted spirals lacking the tight corkscrew appearance of the *Treponema pallidum* (Smith L W and Gault E S 1942 Essential of Pathology ed 2 New York Appleton Fig 1/2)

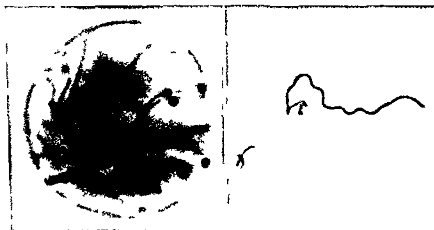


Fig 54 (*Left*) Kazan strain of cultured spirochete Multispirochetal cyst showing development of spirochetes from individual granules (*Right*) Nichols strain of cultured spirochete Late stage in emergence from unispirochetal cyst (DeLamater E D et al Am J Syph *35* 164 216 1951)

hence the derivation of its present name—a *pale fine thread* When suspended in a thin medium such as serum the spirals are regular and angular, from 4 to 14 in number with the depths of the spirals being from 0 5 to 1 μ and the distance between them about 1 μ In this medium the organism has a fairly rapid rotary motion with little or no movement of translation and while difficult to describe, both its morphology and type of movement are distinctive and serve to differentiate the *Treponema* from the *Borrelia* and the *Leptospira*

However in a more viscid medium, such as the mucoid material of the early syphilitic lesion the organism may have a more elongated appearance as though a coiled spring were overstretched and its motion may be writhing undulating and snakelike in character with considerable movement of translation Both morphology and motility can be altered artificially by changing the density of the medium

As demonstrated by electronmicroscopy *T pallidum* in common with most spiral micro organisms has an axial filament about which the specially shaped protoplasm is wound and the protoplasm is encased in a thin periplast Flagellalike structures have been demonstrated but it is not known what role if any they play in locomotion The presence of capsular material probably mucoid in character has been postulated but largely on the basis of indirect evidence rather than by visualization (see Turner and Hollander 1957) Knoblike structures are often seen within the body or attached to the end or the side of the treponeme but it is not known whether these are significant structures in a complicated life cycle of the treponeme as suggested by studies of the Reiter spirochete or merely evidences of degenerative changes For further discussion of this point see the section on Culture Treponemes It is believed that division usually occurs by transverse fission

Treponemes stain readily with many different dyes but because the mass of protoplasm is so slight, contrast is not achieved therefore, from a practical point of view staining methods are unsatisfactory (Campbell and Rosahn 1950) The silver staining method of Levaditi and its many modifications is based on the deposition of metallic silver on the surface of the treponeme thereby increasing the contrast Due to irregular deposition of silver in fixed tissue sections, this stain gives variable and unpredictable results

Cultivation *T pallidum* and other pathogenic species of treponemes have not been grown in vitro despite isolated reports from time to time of successful cultivation Indirect evidence suggests that the pathogenic treponemes are obligatory anaerobes Attempts to grow the pathogenic organism in fertile eggs or in tissue culture have failed thus far

Resistance to Physical and Chemical Agents The survival time of *T pallidum* in vitro is affected by a number of factors Suspensions of organisms adequately freed of serum and tissue extractives and resuspended under anaerobic conditions in a fluid containing crystallized serum albumin dissolved CO either cysteine or glutathione pyruvic acid and a serum ultrafiltrate factor may remain actively motile for 4 to 7 days at 25° C and for 1 to 2 days at 37° C (Nelson 1948 Rice and Nelson 1951) Although supplementation with a number of additional factors further prolongs survival there has been no evidence of multiplication

When frozen at the temperature of dry ice (approximately − 70° C) a significant proportion of the organisms remains motile and infectious for years (Turner and Fleming 1939 Turner and Hollander 1957) The proportion of treponemes that survive the freezing and thawing process is considerably enhanced by the addition of 15 per cent glycerol to the suspending medium (Hollander and Nell 1954) In plasma whole blood or serum stored at refrigerator temperatures the organisms remain viable for 24 hours but not for 48 (Ravitch and Chambers 1942) a fact of importance in relation to the problem of transfusion syphilis The organisms usually die on desiccation even when lyophilized directly from the frozen state However Hampp (1951) has reported that on the desiccation of minced testicular chancre some of the organisms may remain viable up to 66 days In the tissues after death the treponemes may remain infectious for 1 to 5 days In infected animals *T pallidum* apparently may be killed by elevating the body temperature to 41 5 to 42°C and the treponemicidal action of both the arsenicals and penicillin in

velops in 10 to 60 days Two to 12 weeks thereafter a generalized skin rash appears in most patients with organisms demonstrable in the lesions This secondary stage which may also involve the mucous membranes the eyes the osseous system and the central nervous system reflects the generalized dissemination of the organisms and their multiplication at the foci but the evolution reflects the interplay between host and parasite including the development of antibodies

The subsequent course of the disease is extraordinarily varied and depends on the particular tissue involved While approximately 25 per cent of the patients proceed to apparent spontaneous cure and an equal proportion never again have symptoms referable to the disease approximately half of those infected develop late complications varying in severity and prognosis from the relatively benign gummas of the skin and bone to the prognostically serious cardiovascular or central nervous system involvement In the late skin lesions the intensity of the cellular reaction is out of all proportion to the number of organisms demonstrable either by darkfield examination or by animal inoculation strongly suggesting that the tissues have become sensitized to the products of the organisms

In rabbits the disease in its initial stage differs in no important respect from the disease in man Animals may be infected via the eye the skin the testis or the scrotum One spirochete inoculated intratesticularly is regularly infectious and 4 organisms inoculated intradermally cause infection in half the animals (Magnuson Eagle and Fleischman 1947) The time required for the development of a definite inflammatory lesion at the site of inoculation varies with the size of the inoculum with an average linear decrement of 4 days for each 10-fold increase in the number of spirochetes inoculated in the range 1 to 1 000 000 Inoculation of 500 organisms intradermally yields an incubation period of approximately 17 days This suggests a division time in vivo of approximately 30 hours (Magnuson Eagle and Fleischman 1948 Cumberland and Turner 1949 Hollander Turner and Nell 1952) The slow rate at which the organisms multiply in vivo in both man and rabbit is further indicated by the months which may elapse between the completion of inadequate treatment for early syphilis and the appearance of relapsing lesions It is further evidenced by the fact that penicillin in aqueous solution can be administered once daily without prejudicing the outcome of treatment

There are conflicting reports as to the speed with which the organism leaves the site of inoculation and invades the regional lymph nodes and the circulation from as little as 5 minutes to as long as 4 to 24 hours The rate of migration probably varies with the animal species the particular tissue involved its vascularity and lymphatic drainage and the degree of tissue damage incidental to the inoculation

The rabbit infection differs from that of man in several important respects In man a certain proportion of the cases apparently undergoes spontaneous cure in a biologic sense and the lymph nodes even in untreated patients remain infectious for animals in only a small number of instances However the rabbit apparently remains infected for the rest of its natural life Organisms persist in the lymph nodes the spleen the blood and bone marrow and can be demonstrated by inoculating those tissues into normal animals (Frazier Bensel and Keuper 1950 1952) Further while some human cases of syphilis experience spontaneous cure in others there are late complications which may affect almost any organ and in which there may be extensive tissue involvement In rabbits on the other hand once the early stages of the disease have healed there is only a minor microscopic inflammatory reaction even in tissues known to harbor the organisms Although the skin bone and the eyes may be involved in the early inflammatory process late visceral manifestations (heart liver and central nervous system) have been described only rarely

Monkeys and chimpanzees are susceptible to inoculation with *T pallidum* (Metchnikoff and Roux 1903) The early course of the disease parallels that in man and in the rabbit but it is not known whether the animals develop late complications resembling those seen in the human infection Mice and rats were shown by Kolle and Schlossberger to harbor the organisms for long periods without developing gross lesions and a variety of

vivo is enhanced at those higher temperatures Whether the therapeutic action of malaria in cases of neurosyphilis is due solely to the treponemicidal action of the higher temperatures, whether the body s natural defense mechanisms are enhanced at those higher temperatures, and whether antibodies are elaborated in the course of malarial infection which cross react with *T pallidum* remain open questions

The organisms are immobilized rapidly by trivalent arsenicals, bismuth and mercurials There is reason to believe that these compounds are treponemicidal by virtue of their common affinity for —SH groups in the organism and probably because they block essential —SH groups in enzyme proteins vital to the cellular economy Like the immobilization of trypanosomes or the inactivation of certain enzymes the toxic effects of arsenic mercury and bismuth on *T pallidum* are partially reversible Under appropriate experimental conditions organisms immobilized by these compounds may be reactivated by —SH containing compounds such as cysteine glutathione or 2 3 dimercapto propanol These remove the toxic metal from its reversible combination with the cellular constituents Pentavalent arsenicals are inactive in vivo which suggests that the therapeutic activity of these compounds is referable to their reduction to the trivalent compounds in vivo

Penicillin is directly treponemicidal both for the pathogenic *T pallidum* and the cultivated saprophytes The former organism is one of the most sensitive yet studied in terms of the minimal effective concentrations both in vivo and in vitro Paradoxically it is one of the most resistant organisms in terms of the rate of that treponemicidal effect (Eagle Fleischman and Musselman 1950)

Cultivable Treponemes Numerous strains of treponemalike organisms have been cultivated on artificial media These spirochetes are nonpathogenic for experimental animals and presumably for man, and are generally regarded as being not closely related to pathogenic treponemes However they do possess certain common antigenic fractions (D Alessandro and Dardanoni 1953) The Reiter strain has been especially well studied Good growth is obtained in anaerobic broth or thyoglycollate media provided that serum is added more detailed requirements have been delineated by Steinman and Eagle (1950) Various developmental phases in the growth of the Reiter spirochete have been observed by Gelperin (1949) among others

When this organism is maintained in culture for 2 to 3 weeks under adverse conditions balloonlike transparent spheres which contain small round translucent bodies appear at one end of nonmotile spirochetes It has been suggested that these balloons represent an encystment stage

Host Range and Pathogenesis

T pallidum is the etiologic agent of syphilis and in nature is confined to its human host It has proved to be infectious in rabbits and monkeys, in which it causes infections which resemble the human disease in many respects but with important differences In mice and many other rodents the organisms multiply only poorly, without producing a significant tissue reaction, but are demonstrable by subinoculation into rabbits No mammalian species has been found wholly resistant to infection with *T pallidum*

The human infection is usually transmitted by sexual contact In men, the organisms usually are present in lesions on the penis, but they may originate anywhere in the genitourinary tract and be discharged with the seminal fluid In women organisms may derive from mucocutaneous perineal lesions mucous patches on the vaginal wall or from cervical lesions In approximately 10 per cent of the cases the infection may be extragenital usually caused by a chancre or a mucous patch in the mouth a lip or a tonsil It is doubtful that the organisms can penetrate the intact skin however, it is possible that they can penetrate the thinner epidermal layer of the mucous membranes and it is probable that in many persons they gain access through a break perhaps only microscopic in the epidermal layer Although some of the organisms move away from the site of inoculation to reach the adjacent lymph nodes and thence cause a systemic infection within a period of hours to days most apparently remain at the site of infection When they have multiplied to a sufficient degree there is set up a characteristic inflammatory response known as the primary lesion or chancre which usually de-

huson Thompson and Rosenau 1950 Hollander Turner and Nell 1952) The evolution of the immune process may be interrupted at any time by penicillin treatment of the animal Asymptomatic syphilitic infection is not capable of inducing immunity but once immunity is fully developed latent infection may play a role in its maintenance

In the normal evolution of the experimental disease resistance to challenge inoculation reaches its height 2 to 3 months after the beginning of the first infection In the absence of specific treatment it is then maintained at a high level for the remainder of the animal s life Contrary to the course of immunologic events in untreated syphilis when curative treatment is given early during the first infection the development of immunity is interrupted and remains feeble On the other hand when curative treatment is delayed until the third month immunity to challenge inoculation is maintained at a high level for months and even years

Serum Antibodies Concomitant with the early evolution of the initial syphilitic lesion the experimental animal (and man too) develops serum antibodies which may be regarded as specific for treponemal infections although some qualification of this statement will appear below These antibodies are of several kinds and in the light of limited knowledge appear to be induced by antigenic stimuli provided by the infecting treponeme Serum antibodies in syphilis and the serologic tests by which these antibodies are demonstrated can be divided from a biologic as well as a historical standpoint into 2 large groups which may be designated for lack of better terms Wassermann antibody and treponemal antibody

Wassermann Antibody In 1907 Wassermann Neisser and Bruck using complement fixation technics developed by Bordet and Gengou discovered what has become known as Wassermann antibody Since fetal liver rich in *T pallidum* was the first antigen used in the Wassermann test the complement fixing antibody which is commonly present in high titers in patients with early syphilis was regarded as an antibody specific for that organism However oon it was discovered that this antibody could just as readily be demonstrated by using normal liver as antigen and later it was demonstrated that alcoholic extracts of a wide variety of human or animal tissue could serve as the antigen in the Wassermann test The antigen commonly used at present is a highly purified lipid extract of beef heart the so-called cardiolipin to which lecithin and cholesterol have been added (Pangborn 1945)

Much peculation and investigative work have been devoted to the question of whether this antibody which is regularly present in most human beings and animals which have had a treponemal disease is a true antibody to a component of the treponemal organi ms or whether it represents a response to some abnormal tissue lipid component developed during the course of treponemal infection No attempt will be made here to ummarize the evidence bearing on this que tion suffice it to say that the author inclines to the first point of view while equally well informed colleagues favor the second

At any rate for 35 years tests based on the demonstration of Wassermann antibody (or reagin as it is sometimes called) constituted the principal laboratory aid to the clinician in the diagno is of syphilis and related diseases and indeed so they remain today During that period a large number of laboratory tests were developed for the serologic diagnosis of syphilis each usually being denoted by the name of the originator In general two kinds of tests were devised one being based on the principle of complement fixation as indicated above and another utilizing the principle of aggregation of particles in the presence of pecific antibody or flocculation as it is generally known Originally this latter technic was developed by Michaelis

Thus in common use today are complement fixation tests designated Boerner Lukens Eagle Kolmer Wassermann and flocculation tests known as Davies Eagle Hinton Kahn Kline Meinicke Muller Mazzini Rein Bossak VDRL and others (It is historically ironic that the names of Bordet and Gengou and of Michaelis have been almost wholly lost in these relationships) Since these tests represent modifications of the same *basic* principle and detect the same antibody it has become the practice in many places to include them all under the designation serologic tests for syphilis or S T S for short

rodents have been found to undergo a similar asymptomatic infection (Bessemans and de Moor 1939 Wile and Johnson 1945) A small proportion of infected hamsters and guinea pigs develops a lesion at the site of inoculation of *T pallidum* (Turner and Hollander 1957)

Evolution of the experimental disease is influenced by a number of factors, aside from those associated with specific immunity Among the most important of these so called nonspecific factors are trauma temperature and certain hormonal influences

In both experimental and human infections *T pallidum* has been shown to have a special predilection for inflamed or traumatized areas the underlying mechanisms involved are not understood (Chesney and Kemp 1925) There is considerable indirect evidence that in rabbits *T pallidum* multiplies best at a temperature approximating 35 C rather than at body temperature which is normally 38 C or slightly higher In rabbits the incubation period of the initial lesion following inoculation of *T pallidum* into the shaved skin is shorter and the resulting lesions are much larger when the animal is maintained at a cool environmental temperature (18 to 21° C) than at a warm temperature (29 to 31° C) at the latter temperature lesions develop poorly or not at all These differences apparently are not due to changes in the internal body temperature of the animal The same phenomenon is observed in hamsters In both these species there are indications that localization of syphilitic lesions may be influenced considerably by local tissue temperature in rabbits generalized lesions of the skin and bone occur principally on the extremities and about the nose the ears and the tail where the local temperature may be 1 to 3 lower than internal body temperature (Bessemans 1938 Hollander and Turner 1954)

Hormonal factors likewise have been shown to be influential in experimental infections of the rabbit Estrogens tend to make the disease milder and androgens more extensive However the most dramatic effect is produced by the administration of cortisone Given during the early phases of the disease cortisone in doses of 3 to 6 mg per kg body weight per day causes the syphiloma to become softer, more mucoid and to contain tremendous numbers of motile treponemes Withdrawal of cortisone usually results in a rebound phenomenon in which the syphilitic lesions increase rapidly in number and size to produce extensive disease These changes appear not to be due primarily to the effect of cortisone on the production of antibody but on some alteration in the local tissue parasite relationship It is postulated that the treponeme itself is the source of the great overproduction of mucoid material which has been identified as being mainly hyaluronic acid (Turner and Hollander 1952) To what extent these phenomena occur in human infection is not known

Immunity Phenomena in Syphilis

Once a patient has been through the stages of early syphilis he is unlikely again to have primary or secondary syphilis even in the face of repeated exposure to infection Reinfection in syphilis may occur but it is usually symptomless except in patients who were given specific treatment in the early stages of the first infection Clinical and experimental observations indicate that these results can be explained on an immunologic basis

For many years it was believed that immunity in syphilis because of its slow development and its limited effectiveness was qualitatively different from the immunity mechanisms involved in most other infectious processes The extensive studies of Chesney Uhlenhuth Tani Magnuson Turner Nelson and Hardy and their respective co workers provide a body of evidence supporting the conclusion that immunity in syphilis is qualitatively similar to that in most other infections although to be sure knowledge in many areas is deficient especially does the whole phenomenon of latency in treponemal infection and indeed in many other infections remain obscure as to its basic mechanism

Host Reaction In experimental syphilis in the rabbit beginning resistance to a second infection becomes evident about 3 weeks after the appearance of the initial lesion although the rapidity of development and the degree of immunity is clearly related to the number of treponemes in the host (and probably the antigenic mass) as manifested by the number and the extent of the initial lesions (Mag

stimulated the application of other technics for the detection of this or related antibodies Among such technics described have been immune adherence (Nelson 1953) treponemal agglutination (McLeod and Magnuson 1953 Hardy and Nell 1955) complement fixation (Portnoy and Magnuson 1955) and particulate adhesion of the Rickenberg type (La manna and Hollander 1956) None of these technics has been subjected to sufficient clinical evaluation to permit assessment of their clinical or biologic merit although in general the results of these tests show a close parallelism with those of the treponemal immobilization test However there are indications as pointed out below that these tests may not be measuring the same antibody even though ordinarily the antibodies in question may occur together in nature

Antigenic Fractions of Treponemes Reflecting a somewhat different approach application of modern immunochemical methods to the study of fractions of treponemes has revealed the antigenic complexity of these organisms it is probable that knowledge in this area will be extended considerably in the next few years Hardy and Nell (1955) for example working with agglutination technics have described both a heat labile and a heat stable fraction of pathogenic treponemes each of which is capable of inducing its own specific antibody which appears to differ in specificity from the other and from both immobilizing and Wassermann antibody Likewise Portnoy and Magnuson (1955) have prepared by desoxycholate extraction fractions of virulent *T pallidum* which react specifically with syphilitic serum The antibody thus detected appears to parallel fairly closely the occurrence of both immobilizing and agglutinating antibody but its exact relationship to these has not been determined Still another area of investigation which may prove to be of great practical as well as theoretical value has been pursued by D Alessandro and Dardanoni (1953) These investigators have found antigenic components of the nonpathogenic Reiter cultivable treponeme which react specifically with syphilitic serum These components appear to be carbohydrate in nature but their antigenic relationship to various fractions of the pathogenic treponemes has not yet been determined

It should be noted however that while the repeated injection of rabbits with whole killed *T pallidum* or one of its fractions and with large quantities of the Reiter cultivable treponeme or one of its fractions may give rise to a variety of antibodies including Wassermann antibody depending on how the treponeme suspension has been treated it has not yet been possible to induce the development of treponemal immobilizing antibodies except in very low titer This leads to the speculation that both specific immunity and immobilizing antibody are induced by some highly labile antigenic component or components of pathogenic treponemes a fraction possibly associated with the mucoid slime layer of the organism

Suspensions of killed washed and concentrated *T pallidum* have also been used as an intradermal test after the fashion of tuberculin (Marshak and Rothman 1951 Csonka 1955) Positive tests are observed most commonly in patients with late syphilis but the immunologic or clinical significance of these observations has not been shown conclusively

Antibodies in Other Tissues Antibodies in syphilis are like other antibodies associated with the globulin fraction of serum and may be found wherever serum globulins abound For example such antibodies enter the fetal circulation and may be found in cord blood and in the infant for a number of months after birth These antibodies may also be found in the cerebrospinal fluid of syphilitic patients particularly if the blood brain barrier is sufficiently altered to permit easy passage of serum globulins Indirect evidence suggests that active syphilitic disease of the central nervous system may also induce the local formation of these antibodies

Diagnosis

The diagnosis of syphilis in its numerous manifestations rests on (1) clinical observation (2) the demonstration of *T pallidum* usually by darkfield examination of the exudate from an open or abraded primary or secondary lesion and (3) serologic changes in the blood and the spinal fluid

The diagnostic use of the darkfield examination is complicated by the fact that exudates from nonsyphilitic lesions may contain spiral organisms Although most of these can be differentiated readily from *T pallidum* by their larger size coarser spirals and different type of motility a few may be difficult to

In the past few years however there has been a growing awareness that some individuals who never have had syphilis or any other treponemal disease nevertheless have Wassermann antibody in significant amounts in their serum as demonstrated by positive complement fixation or flocculation tests for syphilis Such transient biologic false positive serologic tests for syphilis are not uncommon during the course of many acute illnesses or immediately following certain vaccination procedures More confusing from both a clinical and a biologic standpoint are individuals who carry significant amounts of Wassermann antibody in their serum over periods of months or years and yet according to convincing evidence never have had a treponemal infection Some evidence has been adduced by Moore and Mohr (1952) that the presence of such antibody may indicate the existence of one of the collagen diseases in either an overt or an asymptomatic form

Treponemal Antibodies When syphilitic serum is mixed with suspensions of viable *T pallidum* and these mixtures are then inoculated into rabbits the production of lesions is either delayed or prevented (Tani 1936 Turner 1936 Turner et al 1948) Further when tissue containing *T pallidum* is implanted into the skin of an immune rabbit there is progressive death of the organisms in the implant treponemes are not demonstrable in the adjacent lymph nodes and the implant loses its infectivity after 2 days In normal animals however the treponemes readily penetrate into the lymphatics to cause generalized infection and remain viable in the implant for at least 2 weeks (Tani and Aikawa 1940 Reynolds 1941)

In 1949 Nelson and Mayer produced convincing evidence of the existence in human beings and animals with syphilis of an antibody that reacts specifically with *T pallidum* and closely related treponemes and is separate and distinct from Wassermann antibody Demonstration of this antibody was accomplished through immobilization of *T pallidum* in the presence of complement and has since become known as the Treponemal Immobilization Test or TPI test Its discovery was facilitated by the earlier development by Nelson (see p 388) of methods for the prolonged maintenance of pathogenic treponemes in vitro Simple in principle but difficult of execution, the TPI test utilizes a concentrated suspension of motile *T pallidum* extracted from syphilomas of rabbits testes the human or animal serum to be tested and fresh guinea pig serum containing complement An appropriate mixture of these materials is incubated anaerobically for 18 hours and the test is read by determining under the darkfield microscope the proportion of treponemes which have been immobilized (i e , lost motility and presumably killed) as contrasted with the proportion still motile in tubes containing known normal serum Syphilitic serum repeatedly absorbed with cardiolipin antigen until it no longer contains demonstrable Wassermann antibody will continue to immobilize treponemes in essentially undiminished titer

While technical difficulties have limited the wide use of the TPI test in practice a large number of clinical and laboratory studies have demonstrated its high degree of reliability in reflecting the presence or the absence of prior infection with *T pallidum* or closely related treponemes

During the course of the natural infection in man and the experimental infection in rabbits immobilizing antibody, along with Wassermann antibody can be detected in the blood serum about 1 to 3 weeks after the appearance of the primary syphilitic lesion The titer of immobilizing antibody continues to rise over the next few weeks or months and remains at a high level in untreated persons and animals for many years and probably for life There are some data suggesting that immobilizing antibody tends roughly to parallel immunity to reinfection in syphilis but the evidence on this point is by no means conclusive However it is clear that the titer of Wassermann antibody at least in the experimental animal does not parallel the immune state but rather is correlated with the number and the extent of active lesions For example rabbits 6 months after initial infection commonly show no evidence of active disease and tests for Wassermann antibody are usually negative yet they almost invariably show a high degree of immunity to challenge inoculation and the presence of immobilizing antibodies in their serum

Demonstration of immobilizing antibody

latter so that eventually most of such patients show negative serologic tests In more long standing cases the decline in both types of antibody is much more gradual in many patients tests for Wassermann antibody eventually become negative whereas in most individuals tests for treponemal antibody remain positive It can be argued that the persistence of these antibodies reflects persistence of infection but the weight of clinical and immunologic evidence is against this necessarily being true Just as tetanus toxoid induces specific antibodies that persist for many years so there is much indirect evidence suggesting that both Wassermann and treponemal antibody may persist for many years after elimination of all treponemes by penicillin therapy

Therefore in the present state of our knowledge it would be unwise to assume that individuals who have received what is regarded as adequate treatment for syphilis and still show the presence of these antibodies are still infected Perhaps the best serologic guide to adequate therapy is evidence of some decline in titer of these various antibodies recognizing that such a decline may be detectable only over a period of years and not necessarily over a period of weeks or months

Spinal Fluid Tests Under normal circumstances the serum antibody does not pass into the spinal fluid even in syphilitic patients with a high serum titer In patients with central nervous system syphilis the antibody appears in the cerebro spinal fluid either because of damage to the blood brain barrier or through local elaboration Therefore the presence of a positive test for Wassermann or treponemal antibody in the fluid is usually indicative of involvement of the central nervous system The antibody titer of the fluid coupled with its cell content and protein content and their response to antisyphilitic treatment provide information of diagnostic and prognostic value Tests for treponemal antibody have had less thorough clinical evaluation than have tests for Wassermann antibody

Treatment

Arsenicals notably arsphenamine and its derivatives in conjunction with bismuth and mercury were until recently the drugs of choice in syphilis Now these have been entirely superseded by penicillin which has been shown to be far more active as a treponemicidal agent as well as much less toxic for the patient

First shown to be effective against *T pallidum* by Mahoney Arnold and Harris (1943) the use of penicillin has revolutionized the therapy of syphilis and other treponemal diseases Instead of months and often years of therapy with arsenicals and heavy metals all of which were inherently toxic to the patient curative therapy is now accomplished in most patients within a period of 2 weeks although much longer period of observation are desirable

Patients with early syphilis are treated with a single large dose of a long lasting penicillin 2 4 million units being injected intramuscularly at 2 or more sites Benzathine penicillin G fortified with procaine penicillin G is the preparation of choice although procaine penicillin in oil containing 2 per cent aluminum monostearate in the same dosage is also satisfactory In the small proportion of patients who fail to be cured by this treatment the dose can be repeated In patients with late or latent syphilis the foregoing treatment may be used or the total dosage may be divided into 4 equal weekly injections

Tetracyclines have some treponemicidal action but are far less effective than penicillin

Epidemiology and Control

Syphilis is acquired principally by sexual contact extragenital infection accounting for less than 10 per cent of the total Primary and secondary syphilis in which there are open lesions discharging millions of organisms are the most infectious stages but patients may be infectious for months and occasionally for several years after the secondary lesions have disappeared spontaneously

Infection may be transmitted to the fetus in utero years after initial infection of the mother it is not known whether infection of the fetus occurs through transient spirochetemia in the mother or direct passage of treponemes through the uterine wall

The control of syphilis is made particularly difficult because many infected individuals are not aware that they have the disease this is particularly the case in females in whom the early lesions may be entirely within the geni

distinguish with certainty On the other hand, a negative darkfield does not necessarily exclude syphilitic infection In the late stages of the disease the number of spirochetes is extraordinarily small in relation to the degree of the inflammatory reaction and the demonstration of treponemes whether by staining darkfield examination or even animal inoculation is rarely successful Even in the early lesions failure to detect the organisms in the exudate does not exclude their presence in large numbers Thus if a drop measuring 0 01 ml is placed under a coverslip 22 mm square and examined at a magnification of 900× each field represents approximately 10^{-6} ml of fluid The presence of 1 organism per field then implies the presence of 10^{6} organisms per ml but conversely the absence of visible treponemes in even 100 microscopic fields is compatible with the presence of as many as 10 000 treponemes per ml of fluid Fortunately the number of organisms in the exudate of early lesions is usually so large that 1 or 2 preparations suffice to detect them, when few in numbers the organisms usually can be found by repeated darkfield examinations of the exudate over a period of several days avoiding the use of local antiseptics

Serologic tests constitute the most frequently used method by which a diagnosis of syphilis is made or confirmed Some of the problems and limitations in the serologic diagnosis of syphilis and related diseases have been indicated in the foregoing section By and large these tests when used singly or in combination do provide the clinician with reliable and valuable diagnostic evidence however, their use as guides in the therapeutic management of the patient have greater limitations

During the incubation period of syphilis and for the first 1 to 3 weeks after development of the chancre or the initial lesion all serologic tests are commonly negative From thence forward however with extremely few exceptions patients with clinically overt syphilitic disease show positive serologic tests for syphilis both those that detect Wassermann antibody and those that show the presence of so called specific treponemal antibody

Patients with latent syphilis i e no physical signs of the disease as a rule too have positive serologic tests indeed, in the final analysis a diagnosis of latent syphilis is based solely on such tests It is known however that a small proportion of patients may have negative tests for Wassermann antibody but positive tests for treponemal antibody it is in such cases that the TPI and related tests are valuable Because of the technical difficulties inherent in the TPI test, it is not feasible at the present time to test for treponemal antibody all blood specimens which show a negative test for Wassermann antibody In practice therefore it is customary to test for treponemal antibody specimens from patients in whom the presence of syphilis is suspected on clinical or epidemiologic grounds Improvements in technics and further evaluation of some of the tests for treponemal antibody may eventually permit their substitution for the Wassermann tests in the routine examination of serum specimens for evidence of treponemal disease

At the present time tests for treponemal antibody — TPI agglutination complement fixation—are of greatest practical value as an aid in identifying patients with biologic false positive tests for Wassermann antibody Persons who show positive tests for Wassermann antibody in the absence of clinical or epidemiologic evidence suggesting syphilis should have their serum tested for the presence of treponemal antibody before a diagnosis of syphilis or other treponemal disease is established

Serologic tests have been used traditionally as a guide to therapy in syphilis and related diseases but their interpretation is fraught with difficulty It is a moot question whether antibody production continues for long after the antigenic stimulus provided by the treponeme has been eliminated Data from experimental animals with well established infections indicate that following curative treatment Wassermann antibody declines fairly rapidly but treponemal antibody remains present for long periods although declining gradually in titer

There is some evidence suggesting that the same phenomena occur in human beings on an extended time scale but the data are not conclusive Following successful treatment of early syphilis both Wassermann antibody and treponemal antibody decline over a period of months the former more rapidly than the

medium (see p 522) for several days and virulence is maintained for years when frozen at the temperature of dry ice (approximately −70 C) It does not survive freezing desiccation

T pertenue has the same host range as *T pallidum* and the evolution of the experimental disease in the animal host follows much the same pattern as experimental syphilis However the character of the lesions tend to differ from those of syphilis particularly in that the yaws lesions in general are much less indurated the collections of round cells are more focal and there is strikingly less mucoid material in the lesions In rabbits for example intratesticular inoculation of yaws treponemes commonly gives rise to multiple small focal lesions in the visceral tunic of the testis the so-called granular periorchitis which while it does occur occasionally after the inoculation of *T pallidum* is rare in experimental syphilis Similarly in hamsters the intracutaneous inoculation of *T pertenue* gives rise to a local lesion at the site of injection in the great majority of animal at the same time treponemes are demonstrable in the regional lymph nodes whereas following intradermal inoculation of *T pallidum* the organisms are regularly found in the regional lymph nodes but lesions at the site of injection are uncommon (Turner and Hollander 1957) The basis of these differences is not clear although it may be related in some as yet indefinable way to the observed difference in the amount of hyaluronic acid in the lesions of the two infections (see further discussion of this subject in the section Biologic Relationships Within the *Treponema* Group p 536) Infection with *T pertenue* leads to the induction of both Wassermann and treponemal antibodies in experimental animals and man Generalized lesions in rabbits are less common in yaws than in syphilitic infections The basis for these differences is obscure

The Disease in Man

In man the mother yaw appears 3 to 4 weeks after exposure as a painless yellow red papule (framboise or raspberry) surrounded by an inflammatory zone This gradually increases in size erodes and ulcerates the dried exudate forming a dark crust From 6 weeks to 3 months later sometimes after the mother yaw has healed completely generalized secondary lesions develop which differ in no important respect from the primary lesion When they localize in mucocutaneous junctions (mouth nose perineum) the lesions are moist and resemble syphilitic condyloma Successive crops may appear over a period of several months to several years Plantar papules and hyperkeratosis of the soles of the feet—the so-called crab yaws—are among the most common and the most incapacitating lesions

The late sequelae of yaws are generally restricted to the skin and bone Gummatous nodules and deep chronic ulcerations or crippling bone and joint lesions may develop A destructive ulcerative mutilation of the rhinopharynx (gangosa) a proliferative exostosis of the upper maxilla (goundou) and juxta articular nodules are also ascribed to yaws Visceral complications are rare Although aortic and central nervous system involvement have been described most workers agree that such complications are rare and certainly much less frequent than in syphilitic infection Congenital yaws is believed not to occur

Immunity

In both animals and man one attack of yaws may confer protection against a second attack Immunity develops slowly thus reinoculation in man in the first 3 years of the infection may result in a modified attack but most infected persons are refractory to reinfection after 10 years There is considerable evidence that yaws confers a measure of protection against syphilitic infection In man almost all observers have commented on the relatively small number of cases of syphilis seen in population groups heavily infected with yaws Findlay and Wilcox (1945) produced a syphilitic infection by the subcutaneous inoculation of *T pallidum* into a subject with a clear history of yaws 10 years previously

Likewise there is evidence that human beings who have had syphilis have some degree of immunity to yaws Jalnel and Lange (1926) and Strong (1942) could not induce yaws lesions in persons with general paresis Turner (1936) found that of 10 individuals who presumably had latent syphilis all were refractory to inoculation with yaws trepo-

tal tract and largely symptomless Infected prostitutes constitute especially important sources of infection because of the large number of individuals which they may expose to infection

Several approaches to the control of syphilis have been used with an intensive effort in the past few years although the incidence of the disease seems to be declining in many countries it is not always clear to what factors this decline may be attributed An important factor in control is the early and adequate treatment of the syphilitic patient in order to minimize the length of time during which he remains infectious thereby reducing the number of persons to whom he can transmit the disease Each case is potentially the source of a small outbreak and not infrequently 10 20 or even more cases may be traced through several generations of infection to a single individual The sources of infection should be traced to bring under treatment the infected individual from whom the patient acquired his disease who often is the focal point for many other actual and potential infections The contacts to whom the patient may have transmitted the disease should also be found and followed both clinically and serologically Many will have clinical evidence of the disease by the time they can be brought in for examination Their early and adequate treatment will obviously prevent them from transmitting the infection to others Those contacts who are clinically and serologically normal at the time of the first examination may develop the disease subsequently and should be kept under observation for several months In such persons treatment with a relatively small amount of penicillin may effectively abort an infection not evident at the time of the first examination

Methods of prophylaxis applicable principally to males have likewise been developed as a control measure Calomel ointment applied to the genitalia after sexual exposure has been used for many years (Metchnikoff and Roux 1903) Various other prophylactic measures include thorough washing of the genitalia with soap and water mechanical protection by use of the condom during exposure, and the administration of penicillin by mouth or injection It is probable that each of these measures either alone or in combination with one or more of the others is effective in many instances but fails in others Since the infecting treponeme probably moves beyond the point of inoculation within 1 or 2 hours local prophylaxis must be applied promptly after exposure Perhaps the greatest limiting factors in the efficacy of prophylactic measures are failure of the individual to use them properly because of lack of knowledge or simply neglect to use them at all The striking increase in the incidence of venereal disease in the Armed Forces immediately after the end of World Wars I and II affords adequate evidence of the practical limitations of prophylaxis

Although penicillin has been used successfully to prevent the development of infection in the sex contacts of proved cases of early syphilis its routine prophylactic use (e g in the Armed Forces as a substitute for local chemical prophylaxis) is not practicable The limiting factor is the slow rate at which *T pallidum* is killed by penicillin The single peroral dose which suffices to prevent gonorrhea would be ineffective here and repeated dosages by mouth or parenteral administration could hardly be justified as a prophylactic procedure

TREPONEMA PERTENUE AND YAWS
(Synonyms Framboesia Pian)

History

Yaws has been recognized as a clinical entity for centuries *Treponema pertenue* the causative organism was first identified in the lesions of yaws by Castellani soon after the discovery of *T pallidum* It is not known whether the disease was brought to the West Indies by infected African slaves or was indigenous to both parts of the world Yaws is largely confined to the tropics

Biologic Properties of *T Pertenue*

The organism is indistinguishable from *T pallidum* in many of its characteristics including morphology motility staining properties ability to induce Wassermann and treponemal antibodies in man and experimental animals and susceptibility to the arsenical drugs and penicillin Like *T pallidum* *T pertenue* has not been cultivated on artificial media it can be maintained on Nelson's

infectious cases In some campaigns lower doses have been used with apparent success (Hume and Facio 1955) Bicillin a longer acting penicillin preparation may prove to be even more effective

Because yaws occurs principally among peoples of rural tropical areas where there is limited access to medical facilities attempts at control usually have taken the form of a mass campaign too often these have been one shot campaigns based entirely on mass treatment without adequate preliminary survey or organized follow up To be effective control campaigns should consist of (1) survey of a given area by sanitary inspectors for the purpose of detecting all infectious cases (2) treatment of these cases to render them noninfectious (3) close observation of the community by paramedical personnel in order to discover and treat new infectious cases as soon as they occur An educational program pitched at an appropriate level should be conducted as an integral part of the campaign

TREPONEMA CARATEUM AND PINTA

(Synonyms Mal del pinto Carate Azul)

Pinta is a disease characterized in its later stages by the presence of coalescing depigmented and mottled areas on the wrists hands ankles feet and scalp and hyperkeratoses of the palms and the soles It is prevalent in Mexico and Columbia is encountered in most of the American tropics and recently has been reported from the Philippines Africa India and the islands of the South Pacific The number of cases in Central and South America is now estimated at approximately a million Originally considered a fungus infection its treponemal origin was indicated in 1938 by Saenz Triana and Alfonso who demonstrated the organism in exudates from lesions and in fluid expressed from the adjacent lymph nodes

The organism is morphologically indistinguishable from *T pallidum* Already a confusing multiplicity of names has been applied to it among them *T carateum* *T herrejoni* *T pictor* *T pintae* and *T americanum* Although it has not yet been cultivated on artificial media its regular presence in the lesions and the successful transfer of the disease from man to man (Leon y Blanco 1939) with the recovery of the same organism from the induced lesions seems to establish its causal relationship A rabbit is said to have been inoculated successfully with organisms from a human lesion although 4 rabbits subinoculated from that animal did not develop a lesion a human subject simultaneously inoculated did develop a typical darkfield positive primary lesion in 47 days (Leon y Blanco and Oteiza 1945)

Numerous other attempts to infect rabbits and hamsters have failed although in a few instances motile treponemes have been recovered from the regional lymph nodes of hamsters several weeks after inoculation Infected human beings develop both Wassermann antibody and treponemal antibody that immobilize *T pallidum* (Varela and Olarte 1950) Although *T carateum* undoubtedly belongs to the group of treponemal organisms the limits of its biologic relationship to *T pallidum* and *T pertenue* are still vague

The disease may be contracted at any age and is first evidenced by a nonulcerating primary lesion This is followed in 5 to 18 months by the appearance of successive crops of flat erythematous and hyperpigmented lesions (pintid) After several years one observes the characteristic late depigmentation and hyperkeratoses Although there were at first thought to be no late visceral complications Saenz has reported both cardiac involvement and central nervous system involvement

Human beings have been successfully inoculated with the exudates from the lesions but reinfection does not succeed in the late cases Syphilitic subjects have been successfully inoculated with pinta and subjects with pinta may contract syphilis (Herrejon 1940) indicating at least some immunologic differentiation between the two infections

Transmission is not venereal but usually occurs by person to person contact Flies (*Hippelates*) allowed to feed on serous fluid containing the treponemes have been shown to be capable of transmitting the disease to man (Blanco and Parra 1941)

The disease responds to treatment with arsenicals bismuth and penicillin as do yaws and syphilis Penicillin is the drug of choice administered in the same dose and in the same manner as for syphilis

nemes as were 9 of 10 other individuals who had yaws in the remote past, while darkfield positive lesions were induced by the same inoculum in 9 of 10 patients who had had recent yaws infection

There is also a large body of evidence indicating the existence of a degree of cross immunity in the experimental infections When rabbits are infected with *T pallidum* and the disease is permitted to evolve to the point of latency a majority of the animals develop no lesion upon challenge inoculation of yaws treponemes Likewise yaws infected animals which have had the disease 3 months or longer commonly show some degree of immunity upon challenge inoculation with *T pallidum* On the whole syphilis animals exhibit evidences of a higher degree of immunity to yaws than yaws animals to syphilis while the results vary according to the experimental methods employed taken as a whole published data provide incontrovertible evidence of a reciprocal cross immunity between yaws and syphilis For a review of pertinent experiments see Turner and Hollander 1957 Treponemal antibodies occurring during yaws infection in both man and animals have the capacity to immobilize syphilis treponemes and serum from syphilitic animals and man regularly immobilize yaws treponemes (Khan Nelson and Turner, 1951)

Diagnosis

As in syphilis the diagnosis of yaws depends upon (1) the appearance of the lesions (2) demonstration of treponemes in early lesions by darkfield examination and (3) serologic tests The florid skin lesions of yaws and the plantar lesions can scarcely be confused with any other disease Darkfield examination and serologic tests assist in differentiating yaws from nontreponemal disease but not of course from syphilis to other treponematoses

Epidemiology

Yaws is virtually limited to tropical areas and occurs principally where the rainfall is high In some areas fully 75 per cent of all individuals have had yaws by age 20 Areas of high endemicity frequently lie within a few miles of communities in which yaws is uncommon The disease occurs principally among the lower socioeconomic groups of the population living under poor hygienic conditions, but in areas of high endemicity well to do families may be affected

The disease is spread by direct contact with an open lesion discharging treponemes The organism apparently cannot pass through the intact epithelium and a cut or an abrasion perhaps only microscopic, probably serves as a portal of entry The role of flies as a vector has been suggested by Kumm and Turner (1936) who found that a fly (*Hippelates pallipes*) widely distributed in the West Indies fed in large numbers on the open ulcerative lesions and that *T pertenue* could then be found in the foregut or the stomach where it remained viable for about 7 hours The regurgitation of infective material on a breached area of the skin has been shown to cause infection in rabbits *T pertenue* has also been reported to pass through the intestinal canal of certain species of African flies and mosquitoes in viable form Although man can be infected at any age more than two thirds of the infections occur before the age of puberty, and males are infected more commonly than females There is no convincing evidence of variation in susceptibility according to race

Treatment and Control

Yaws responds to the same therapeutic agents as syphilis There is some clinical evidence to indicate that smaller amounts of these drugs are required in yaws than in syphilis but there are no carefully controlled clinical or experimental studies to support this view The trivalent arsenicals the heavy metals such as bismuth and mercury and various antibiotic drugs including penicillin tetracycline and oxytetracycline all have therapeutic action Penicillin is by far the most effective of all known therapeutic agents and is the drug of choice in treatment

Based on experience gained in many campaigns the WHO Expert Committee on Venereal Infections and Treponematoses recommends a single injection of 1 2 million units of procaine penicillin in oil with 2 per cent aluminum monostearate (PAM) for adults with active yaws This dose is reduced proportionately for younger age groups for patients with latent yaws and for contacts of

genitalia they are also found in lung abscesses and in so-called tropical ulcers of the lower legs and the feet although here their etiologic role is not clear

The *Borrelia* group of spirochetes are distinguished from *Treponema* and *Leptospira* by being longer as a rule with the pirals deeper more loosely wound and more flexible These organisms stain well with ordinary aniline dyes but are not readily cultivable on artificial media The type pecies of the human rela ping fever pirochetes is *Borrelia recurrentis* many other species that affect man have been identified and named but as pointed out below it is questionable whether these should be regarded as separate species or as variants of the one type pecies *B gallinarum* is the type pecies of the fowl spirochetoses and *B incenti* of the spirochetes that occur in debilitated tissue lesions of human beings

BORRELIA RECURRENTIS AND RELAPSING FEVER

History

In the 18th century relapsing fever was sometimes confused with typhus fever but the two were clearly distingui hed by Henderson in 1843 The spiral organi m cau ing the disease *B recurrentis (obermeieri)* was first een in the peripheral blood of a human patient by Obermeier in 1868 and the disease was reproduced in man by the injection of infected blood in 1874 In 1904 Ross and Milne showed that so-called African tick fever was also caused by a spiral organism demon trable in the blood and they indicated its probable identity with relapsing fever Shortly thereafter it was shown that as had been su pected the body louse could also act as a vector

Morphology

B recurrentis is a highly flexible spiral organism varying in length from 8 to 30 μ in thickness from 0 3 to 0 5 μ with 5 to 10 irregular and loosely wound spirals which average 1 to 2 μ in depth and 3 μ in width (Fig 56) The organisms are actively motile with both rotational and transitional move ment However the latter is not always pro gressive and in blood preparations the organ isms may move back and forth within the same microscopic field Unlike *T pallidum* *B recurrentis* takes the usual bacterial stains, as well as Wright s Giemsa and other stains containing the aniline dyes The electron microscopic studies of Swain (1955) show a central axial filament with a surrounding sheath of protoplasm Upon washing in distilled water this sheath disappears and nu merous fibrils resembling flagellae are seen These fibrils may arise from the axial filament or be the remains of the sheathlike envelops Division is believed to be by transverse fission

Cultivation and Biologic Properties

Reportedly the organi m has been culti vated on media enriched with serum or blood but these reports are not readily confirmed At the present time the relapsing fever pirochetes cannot be cultured successfully on artificial media whereas they do grow well in the chick embryo (Oag 1940) and direct isolation from the blood of patients has been accompli hed by thi method (Bohls et al 1940) Inoculation can be made onto the chorio-allantoic membrane or into the body of the embryo When planted on media con taining blood serum or ascitic fluid and main tained at refrigerator temperature relapsing fever pirochetes may remain motile and in fective for many months (Wolman and Wol man 1945) Their virulence is maintained for long periods at the temperature of dry ice (Turner and Fleming 1939)

The widespread distribution of the disease and the several modes of transmission have led to the isolation of a large number of strains differentiated primarily on the basis of the area of isolation or the vector con cerned in their transmission rather than by inherent biologic differences in the organisms Although some strains apparently will grow preferentially in certain species of ticks this may not reflect a genetic difference Louse borne strains have been shown to be infective for ticks which then can transmit the disea e to man and naturally tick borne strains have been similarly transferred to lice Adler and Ashbel (1942) have demonstrated the trans missibility of tick borne relapsing fever (*S persicus* in Palestine) to lice but they question the epidemiologic importance of the unnat ural vector

Although agglutinins and other antibodies can be produced in high titer the serologic

TREPONEMA CUNICULI AND RABBIT SYPHILIS

Treponema cuniculi was identified by Bayon in 1913 as the cause of a natural infection of rabbits first described by Ross (1912). The organism is morphologically indistinguishable from *T. pallidum*, which it resembles also in its susceptibility to arsenicals and penicillin. The natural lesion consists of superficial scaly eroded lesions on the genitalia and the adjacent perineal region. Inoculation into the skin of that area reproduces the disease, and intratesticular inoculation causes, in 14 to 28 days, an inflammatory reaction resembling that caused by syphilis but less extensive, lacking the induration characteristic of the latter, and consisting largely of fine nodules in the parietal layer of the tunica vaginalis without marked enlargement of the testis (McLeod and Turner, 1946). In the following 1 to 6 months metastatic lesions may be seen in the testis, scrotum, anus, prepuce and glans, and secondary lesions in the skin and the mucocutaneous borders of the eyes, nose and mouth. Wassermann antibody develops in the same proportion and to the same degree as in rabbits infected with *T. pallidum* and *T. pertenue*. Treponemal antibody to *T. pallidum* also develops but apparently in lower titer than to *T. cuniculi* (Khan et al., 1951).

OTHER TREPONEMATOSES OF MAN

In many geographic areas of the world, particularly where the majority of people are economically depressed and live under unhygienic conditions, endemic treponemal infections are observed. Transmission is commonly nonvenereal, occurring through direct bodily contact or through the medium of common eating and drinking utensils. These nonvenereal treponematoses have often been given local names such as bejel in Syria and adjacent areas where it is endemic among nomadic tribes, siti in British West Africa, dichuchwa in Bechuanaland, njovera in Southern Rhodesia, and endemic syphilis in certain sections of the Balkans. While the clinical and epidemiologic patterns of these syndromes differ somewhat from the standpoint of causative treponemes, serologic tests and response to therapy, they cannot be distinguished from one another or from yaws and syphilis.

BIOLOGIC RELATIONSHIPS WITHIN THE TREPONEMA GROUP

On the basis of comparative in vitro and in vivo studies of 70 strains of treponemes derived from human beings with various treponemal syndromes in many parts of the world, Turner and Hollander (1957) concluded that all strains were closely related in their essential biologic characteristics, in the disease picture they induced in man and in experimental animals, in their immunologic features and in their reaction to antibiotics. However, certain relatively stable differences were observed, particularly in the lesions in rabbits, the disease picture in hamsters, and certain immunologic patterns as determined by challenge inoculation of rabbits.

On the basis of these criteria, strains of treponemes from various parts of the world were placed into one of three categories: (1) syphilislike, (2) yawslike and (3) an intermediate group which partook of some of the characteristics of both syphilis and yaws. Most of the strains from the nonvenereal treponematoses, including some of the yaws strains, belonged to this intermediate group.

These groupings were believed to reflect fundamental biologic differences in the strains of treponemes, and it was postulated that the differences resided in the character and the amount of capsular mucopolysaccharide that each strain produced. Six strains of *T. cuniculi* did not clearly fit into any of the above three categories and may perhaps be regarded as a fourth group.

THE BORRELIA

Introduction

The genus *Borrelia* comprises several species of spirochetes which are morphologically similar but exhibit widely different pathogenic proclivities and host range. The most important members of the group from a medical standpoint are those causing relapsing fever in man; closely related organisms cause a somewhat similar spirochetosis in fowls. These organisms are primarily blood parasites. Another large and perhaps heterogeneous subgroup are the *Borrelia* associated with ulcerative conditions of the oral cavity and the

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INTRODUCTION

The genus *Borrelia* comprises several species of spirochetes which are morphologically similar but exhibit widely different pathogenic proclivities and host range The most important members of the group from a medical standpoint are those causing relapsing fever in man closely related organisms cause a somewhat similar spirochetosis in *fowls These* organisms are primarily blood parasites Another large and perhaps heterogeneous subgroup are the *Borrelia* associated with ulcerative conditions of the oral cavity and the

antigenic variation in successive relapses has been observed even after single cell inoculation (Schuhardt and Wilkerson 1951) However it must be noted that Ashbel (1942) has observed relapses without demonstrable changes in immunologic reactivity and Stein (1944) has furnished evidence of a common antigen in a number of supposedly different strains isolated from both animals and man

Recovered patients have been found to resist reinfection from 2 to 5 years later However other workers have found that patients remained immune only so long as the organisms persisted in the tissues Attempts to immunize animals with killed spirochetes have been unsuccessful and it has not yet been possible to characterize and differentiate strains on the basis of their antigenic reactivity There is evidence of cross protection betwe n tick borne and louse borne strains Monkeys infected with the tick borne California strain proved to be re istant to reinfection with a louse borne Chine e strain and in hamsters when the order of inoculation was reversed the second infection consisted of a single short attack with no relapses (Chen Zia and Anderson 1945)

Diagnosis

The diagnosis rests primarily on the demonstration of the organism in the blood by direct darkfield observation by the examination of stained blood films or by animal inoculation Young white rats weighing from 30 to 80 Gm are particularly susceptible organisms appearing in the blood in large numbers in 24 to 72 hours When one centrifuges the blood of infected animals or man the organisms tend to concentrate in the white blood cell layer facilitating their detection by darkfield examination or stain In louse borne infections Proteus OXK agglutinins in high titer appear in a substantial proportion of the cases (Zarofonetis et al 1946 Davis 1948)

Treatment

The trivalent arsenical drugs particularly neoarsphenamine were used for many years but now have been superseded by penicillin and the tetracyclines However few well controlled clinical or experimental studies have been made with these drugs

Penicillin appears to be effective when given in large doses—1 000 000 units of crystalline penicillin per day in aqueous solution in divided doses every 3 hours for at least 5 days Failures have been attributed to inadequate dosage However the treatment of choice is chlortetracycline in doses of 0 5 Gm every 6 hours for 5 days then 1 0 Gm twice daily for another 5 days Children under 10 should be given half this dose Oxytetracycline in approximately the same dosage as chlortetracycline seems to be equally effective on the basis of limited trials Streptomycin also seems to have some therapeutic value Exacerbation of symptoms (Herxheimerlike reaction) frequently occurs immediately following the initiation of drug therapy (Cherry 1955)

Epidemiology and Prevention

Relapsing fever is transmitted from man to man from animal to animal and from animal to man entirely by insect vectors While many arthropod insects are capable of natural infection transmission to man is primarily by the body louse and by a variety of ticks and the epidemic pattern is determined largely by the prevalence of these vectors and opportunities for man to be parasitized by them Thus great epidemics of louse borne relapsing fever have occurred one of the most recent being the great epidemic wave that swept over North Africa in 1942 44 Louse borne relapsing fever may occur concomitantly with louse borne typhus fever leading to considerable diagnostic confusion Tick borne relapsing fever is largely endemic in occurrence although its prevalence in some areas notably central Africa has reached almost epidemic proportions at times

In northern and western Africa in Europe and in parts of Asia the disease is spread primarily by the body louse *Pediculus humanus* which becomes infectious 4 to 5 days after the ingestion of infected blood and remains infectious for 2 to 3 weeks The infection in lice is not transmitted to the second generation (Wolman and Wolman 1945) The disease apparently is transmitted when lice are crushed near a bite or scratch which provides a portal of entry for the organism It is the louse borne infection which may become epidemic under conditions which lower the host resistance and favor the rapid multi

differentiation of the various strains is complicated by the fact that their antigenic structure apparently changes repeatedly during a single infection It has been suggested that the antibodies first elaborated act as a selective factor which permits the survival only *of antigenically distinct mutants and that the* characteristic relapsing course of the disease is due to the multiplication of these resistant mutants for which the host must now elaborate new antibodies (Cunningham 1925 Meleney 1926 1928 Schuhardt and Wilkerson 1951)

Pathogenesis

The louse and tick borne infections cannot be distinguished with certainty on the basis of their clinical manifestations The disease in man usually begins with an acute febrile onset from 3 to 10 days *after infection In* this initial febrile stage there are usually large numbers of organisms in the blood they may be found in the urine in approximately a fourth of the cases and by animal inoculation sometimes spirochetes can be demonstrated in the cerebrospinal fluid After an average of 4 days the fever declines coincident with the disappearance of organisms from the blood *As the number of organisms decreases they* become less motile tend to assume bizarre forms and may agglutinate in rosettes During the afebrile period the blood is not infectious for lice The afebrile period may last from 3 to 10 days and is followed by a second febrile attack during which organisms reappear in the blood but in smaller numbers There may be from 3 to 10 such recurring febrile attacks The mortality *in the endemic* infection varies between 2 and 5 per cent but in epidemics it may be 50 per cent or even higher In fatal cases organisms are found in sections of the spleen and the liver and are particularly numerous in the malpighian bodies of the spleen which show miliary necrotic lesions Hemorrhagic lesions may be found in the gastro intesitnal tract and in the kidney There have been occasional reports of relapsing fever in the offspring of infected mothers presumably caused by transplacental inoculation

Monkeys mice and rats can be inoculated subcutaneously intravenously *or intraperitoneally* Guinea pigs are susceptible to the tick borne strains the organisms surviving in the brain for more than 3 years (Sergent 1945), while louse borne strains are only rarely infectious on direct inoculation into this species (Coghill and Gambles, 1948) Rabbits and dogs are not susceptible Monkeys show the characteristic relapsing course of the human disease Organisms appear in the blood in 24 to 48 hours and disappear spontaneously in 2 to 5 days In young rats (40 to 80 Gm), *they may appear in the blood in* huge numbers within 24 to 72 hours and disappear with extraordinary rapidity within a few hours Even in these animals however, the disease is rarely fatal Consistent with the demonstration of the organisms in the cerebrospinal fluid and the brain in human cases there is some evidence that in rats mice and guinea pigs the brain may serve as a reservoir *of infection after the apparent disappearance* of organisms from the circulating blood (Heronimus 1928 Anderson 1946 Schuhardt and Hemphill 1946)

Immunity

Serum agglutinins and bactericidal antibodies are readily demonstrable in both the experimental and the human disease and the *termination of the individual febrile* attacks as well as the eventual cure may be related to their appearance It is significant that the organisms which appear during a febrile relapse often differ in their serologic reactivity from those present in the immediately preceding attack (Cunningham 1925 1926 Cunningham and Frazier 1934) In rats a protein deficient diet has been found to cause a significant increase in the severity of infection and mortality (Guggenheim et al 1951) In squirrels inoculated with a single human strain Meleney isolated 6 serologically distinct strains during a series of relapses There is no fixed order in which *the mutant strains* appear in the course of a single infection but there is an indication that the organisms in alternate relapses may be more closely related in their antigenic structure than is the intervening mutant Therefore the development of mutant strains not susceptible to the antibody previously elaborated may be the actual cause of the relapse and final cure may reflect an increasingly broad immunity afforded by the multiple mutant strains The

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plication and the wide dissemination of the insect vector Thus epidemics generally have occurred in malnourished overcrowded populations with poor personal hygiene and often have been incidental to famine and war The seasonal incidence with increased spread in cold weather probably reflects the heavier louse infestation and thickly clothed persons crowding together for warmth

In the endemic areas of Central and South Africa over a wide area of Asia and in the Americas the disease is tick borne The disease is by no means uncommon in the United States In Texas alone 100 cases were diagnosed during the period June, 1942, to May 1949, and it seems likely that many additional cases escaped detection The most important vector is the genus *Ornithodoros* many species of which have been shown to be infected in nature (Table 49) There appears to be a remarkable species specificity in this host parasite relationship There is some evidence that rodents may serve as natural reservoirs of infection for the tick Organisms are found in all parts of the infected tick may persist for years and are transmitted to the ova for many generations their infectivity for mice remaining unchanged However the percent

TABLE 49 GEOGRAPHIC DISTRIBUTION OF VARIOUS SPECIES OF *Borrelia* RESPONSIBLE FOR RELAPSING FEVER AND THEIR VECTORS

		Mode of Transmission	
	Species of *Borrelia**	Louse borne (*Pediculus humanus*)	Tick borne (*Ornithodoros*)
Europe	*recurrentis*	+	
	obermeieri	+	
	hispanica		*O erraticus (marocanus)*
			O verrucosus
Africa	*duttoni (crociduri)*		*O moubata*
	kochi		*O erraticus (marocanus)*
	russi		
	berbera	+	
	aegyptica		
	turicatae		*O turicata*
	marocana		
	sogdiana		*O savignyi*
Middle East	*persica*		*O papillipes (tholozani)*
			O asperus
			O lahorensis
India	*carteri*	+	*O tholozani*
			O crossi
			O lahorensis
Russia	*latyshevi*		*O verrucosus*
			O neerensis
			O tartakovskyi
			O tholozani
North America	*novyi*		
	turicatae		*O turicata*
	parkeri		*O parkeri*
	hermsii		*O hermsi*
Central and South America	*dugesii*		*O dugesi*
	venezuelense		*O venezuelensis (rudis)*
	neotropicalis		*O talaje*
	turicatae		*O turicata*

* Validity of identification as separate species questionable

age of transovarian transmission varies widely in different tick species (Davis 1939 1943 1948) Some tick species appear to transmit infection to man through the coxal fluid while others introduce the organisms directly by a bite The tick borne disease is not usually epidemic and the seasonal incidence probably is related to the prevalence of the vector Infected ticks (*O turicata O hermsi O parkeri* and *O talaje*) have been found over a wide area of the United States (Wyoming Montana Idaho Texas Kansas California Colorado Arizona Utah Florida New Mexico and Oklahoma) Ground squirrel and prairie dog burrows are sometimes heavily infested with ticks with a high incidence of spirochetes suggesting that these and other rodents may serve as natural reservoirs of infection

Although the causative organisms cannot pass through the intact skin they can penetrate the mucous membrane (e g conjunctiva) and transplacental infection may also occur The possible role of the bedbug in transmission has been suggested The risk of infection may be reduced by personal cleanliness and by avoiding contact with persons carrying lice or ticks No effective vaccine is available and chemoprophylactic measures are not practicable In louse borne epidemics the isolation of patients large scale treatment and the mass delousing of the population as with DDT are effective procedures However DDT resistant strains of lice have developed in many areas and this form of control is now less dependable

In endemic areas reduction of the tick population by periodic spraying of living quarters with benzene hexachloride a chemical long used in tick control of cattle has been reported to effect a major reduction in the morbidity of the disease (Holmes 1953)

BORRELIA VINCENTI AND ULCERATIVE LESIONS OF THE OROPHARYNX THE GENITALIA AND THE EXTREMITIES

In a diverse group of infections (tropical ulcer Vincent s angina or ulcerative stomatitis pulmonary spirochetosis ulcerative lesions of the genitalia) one finds large numbers of a delicate short (5 to 10 μ) actively motile spiral organism with a variable number of shallow irregular turns It is usually present in association with a coarse thick gram negative rod with tapered ends which is often banded or beaded in stained preparations (*Bacillus fusiformis*) There is no agreement as to whether these two organisms are the actual cause of the necrotic lesions or only secondary invaders Thus the infection has been ascribed by some to a herpeslike virus (Black 1942) and by others to a predisposing vitamin or other dietary deficiency which would permit the invasion of the tissues by normally harmless organisms of the surface of the mucous membranes It should be noted that these same organisms can be recovered from about the gingival margins of almost all healthy adult human beings sometimes in large numbers in which case there is usually an associated pyorrhea

These organisms appear to respond satisfactorily to the tetracycline group of drugs particularly chloramphenicol and oxytetracycline

LEPTOSPIRA AND THE LEPTOSPIROSES

Introduction

The genus *Leptospira* comprises a group of spiral organisms which in morphology, biologic characteristics and antigenic structure have little in common with either the *Treponema* or the *Borrelia* group of spirochetes Unlike the *Borrelia* and the pathogenic *Treponema* they can be cultivated readily on artificial media A number of species of *Leptospira* have been identified each with fairly distinctive characteristics in respect of antigenic structure and often their host range but all the leptospira cause an acute illness in man which while it may vary in severity is similar in its general pattern *regardless of the* species involved It appears too that wild rodents are the natural hosts and essential reservoirs of all the pathogenic leptospiral organisms Spirochetes with the morphology of leptospira may rarely be found about the gingival margins and in other chronic ulcerative lesions and leptospira (*L biflexa*) have been cultured from tap water but little is known about the antigenic structure of these presumably nonpathogenic varieties

The historical association of jaundice with leptospiral infection stemming from the classic description of the disease by Weil and desig

plication and the wide dissemination of the insect vector Thus epidemics generally have occurred in malnourished, overcrowded populations with poor personal hygiene and often have been incidental to famine and war The seasonal incidence with increased spread in cold weather probably reflects the heavier louse infestation and thickly clothed persons crowding together for warmth

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			O verrucosus
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	kochi		*O erraticus (marocanus)*
	russi		
	berbera	+	
	aegyptica		
	turicatae		*O turicata*
	marocana		
	sogdiana		*O savignyi*
Middle East	*persica*		*O papillipes (tholozani)*
			O asperus
			O lahorensis
India	*carteri*	+	*O tholozani*
			O crossi
			O lahorensis
Russia	*latyshevi*		*O verrucosus*
			O neerensis
			O tartakovskyi
			O tholozani
North America	*novyi*		
	turicatae		*O turicata*
	parkeri		*O parkeri*
	hermsii		*O hermsi*
Central and South America	*dugesii*		*O dugesi*
	venezuelense		*O venezuelensis (rudis)*
	neotropicalis		*O talaje*
	turicatae		*O turicata*

* Validity of identification as separate species questionable

TABLE 50 SEROLOGIC CLASSIFICATION OF SOME OF THE LEPTOSPIRAL SPECIES PATHOGENIC FOR MAN*

SEROGROUPS	IMMUNOLOGICALLY RELATED SPECIES	ISOLATED Year	ISOLATED Country	KNOWN RESERVOIRS	KNOWN DISTRIBUTION
†*L. icterohaemorrhagiae*		1914	Japan	Rats mice other rodents swine foxes apes dogs cats horses poultry calves	World wide
†*L. canicola*		1931	Holland	Dogs golden hamsters	World wide
	L. salinem	19 3	Dutch East Indies	Rats guinea pigs	Southeast Asia
	L. ballum	1943	Denmark	Mice white rats guinea pigs	North America Denmark France
L. hebdomadis		1918	Japan	Field mice dogs	Japan Eastern Asia Indonesia Europe North America
	L. sejroe	1937	Denmark	Field mice	Central Europe Indonesia
	L. saxkoebing	1942	Denmark	White rats field mice	Central Europe
L. pomona		1937	Australia (Queensland)	Swine cattle dogs	Australia Indonesia Middle East Central Europe North and South America
	L. bovis	1948	U.S.A.	Cattle goats sheep	U.S.A., Europe Israel
L. grippotyphosa		1928	Russia	Field mice other rodents	South and East Europe Africa Israel Southeast Asia
	L. bovis (*palestinense*)	1947	Palestine	Cattle goats sheep field mice chickens	U.S.A. Europe Israel
	L. geffe	1950	Israel	Goats cattle voles mice	Israel
L. autumnalis		1925	Japan	Field mice rats dogs guinea pigs	Japan Southeast Asia Indonesia U.S.A. Switzerland
	Fort Bragg Strain	1952 (1943)	U.S.A.		U.S.A.
L. australis		1937	Australia (Queensland)	Field rats shrew dogs swine guinea pigs	Australia Southeast Asia Japan Central Europe
	L. pyrogenes	1922	Indonesia	Field rats guinea pigs bandicoat, swine	Australia Southeast Asia, Italy North America
L. mitis		1940	Australia (Queensland)	Cattle swine	Switzerland Italy Australia
	L. hyos	1944	Argentine	Swine cattle horses	Argentine France
L. bataviae		1926	Netherland East Indies	Rats cats field mice guinea pigs swine dogs	Indonesia Malaya Europe Central Africa

* Compiled largely from Schlossberger and Brandis 1954
† These two subgroups are closely related serologically

nation of the first pathogenic species isolated as *L. icterohaemorrhagiae* probably has contributed to the tardy recognition, in the United States at least, of the true prevalence of leptospiral infections. Jaundice, while a prominent clinical sign in many severe cases, is present *in less than half of all recognized cases of* leptospirosis.

History

Spirochetal fever (also termed "spirochetal jaundice" or "Weil's disease") was first described in 1886 as a febrile disease associated with jaundice and characterized by involvement of the kidney and the spleen. The causative organism was isolated in 1915 by Inada and his co-workers, who also demonstrated the role of rats as natural vectors, inoculated guinea pigs from infected rats and demonstrated *the organism in each stage of that* cycle.

It has since developed that there are many different leptospiral species which vary in their geographic distribution, host range and antigenic structure. Many and perhaps all of these are pathogenic for man, and over the *past 2 decades a number of previously obscure* infections have been identified as leptospiral in origin. The "marsh" or "field" fever of central Europe (*L. grippotyphosa*), the 7-day fever of Japan (*L. hebdomadis*) and "swineherd's disease," first seen in Australia and since recognized in both Europe and the United States (*L. pomona*), *are cases in point.* A significant proportion of cases of so-called aseptic meningitis have also been found to be leptospiral rather than viral infections (Beeson and Hankey, 1952). A recent addition to this list and one of the most unexpected, is "Fort Bragg fever" or "pretibial fever," first encountered *at Fort Bragg, North Carolina (Bow*dom, 1942; Daniels and Grennan, 1943). It was then thought to be a self-limited virus infection, but following several years of serial passage through guinea pigs and hamsters it was discovered by Gochenour and his associates (1952) that the infectious agent so propagated was not a virus but a strain of leptospira not previously encountered in the United States. This strain differs serologically from *L. icterohaemorrhagiae* and *L. canicola* but resembles *L. autumnalis* originally isolated in the Far East.

The continuing identification of leptospira as the etiologic agent in a number of obscure infections, coupled with the protean and often *minimal clinical aspects of the disease,* indicate that human leptospirosis, already known to be world-wide in distribution, *is* probably far more common than has hitherto been suspected, and that the possibility of its presence should be duly considered in infections of obscure etiology. At least 7 antigenically distinct strains are known to exist in North America. All have been isolated from cases of *human infection as well as from the animal* species, which seems to be its more usual host (Yager and Gochenour, 1952).

Morphology and Cultivation

All the pathogenic leptospira are indistinguishable morphologically and in most other *biologic characteristics except antigenic struc*ture. The description which follows will refer specifically to the type species *L. icterohaemorrhagiae* but applies to other species unless otherwise noted.

L. icterohaemorrhagiae is characterized by its extraordinarily fine spirals, so closely wound and so short that *they may be visible* on darkfield examination only as a series of small dots and usually are not distinguished in stained films. The length of the organism varies from 4 to 20 μ. It is approximately 0.1 to 0.2 μ in width and moves by the active rotation of one end of the organism bent into a hook. Both ends may be motile, *in which* case the actively rotating organism has no translational motion. Considerable confusion and mistaken diagnoses in the past originated in the strands which under in vitro conditions frequently extend from the surface of red blood cells and break off to float free in the *plasma. While not actually spiral in form they* are extremely difficult to distinguish under the darkfield microscope from leptospira, even in expert hands; therefore identification of leptospira in human blood specimens is unreliable and never should serve as the basis for diagnosis of the disease.

Electron microscope studies (Czekalowski and Evans, 1955) show a central cylindrical core containing focal areas of increased density which may be nuclei. This core is wound in helical fashion around a much smaller but more rigid axis. *Knoblike structures often*

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†*L. canicola*		1931	Holland	Dogs, golden hamsters	World wide
	L. salinem	19[illegible]3	Dutch East Indies	Rats, guinea pigs	Southeast Asia
	L. ballum	1943	Denmark	Mice, white rats, guinea pigs	North America, Denmark, France
L. hebdomadis		1918	Japan	Field mice, dogs	Japan, Eastern Asia, Indonesia, Europe, North America
	L. sejroe	1937	Denmark	Field mice	Central Europe, Indonesia
	L. saxkoebing	1942	Denmark	White rats, field mice	Central Europe
L. pomona		1937	Australia (Queensland)	Swine, cattle, dogs	Australia, Indonesia, Middle East, Central Europe, North and South America
	L. bovis	1948	U.S.A.	Cattle, goats, sheep	U.S.A., Europe, Israel
L. grippotyphosa		1928	Russia	Field mice, other rodents	South and East Europe, Africa, Israel, Southeast Asia
	L. bovis (*palestinense*)	1947	Palestine	Cattle, goats, sheep, field mice, chickens	U.S.A., Europe, Israel
	L. geffeni	1950	Israel	Goats, cattle, voles, mice	Israel
L. autumnalis		1925	Japan	Field mice, rats, dogs, guinea pigs	Japan, Southeast Asia, Indonesia, U.S.A., Switzerland
	Fort Bragg Strain	1952 (1943)	U.S.A.		U.S.A.
L. australis		1937	Australia (Queensland)	Field rats, shrew, dogs, swine, guinea pigs	Australia, Southeast Asia, Japan, Central Europe
	L. pyrogenes	1922	Indonesia	Field rats, guinea pigs, bandicoat, swine	Australia, Southeast Asia, Italy, North America
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* Compiled largely from Schlossberger and Brandis 1954

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occur at the ends of the axis but it is not known whether these have any function in the reproductive process it is believed that division is usually by transverse fission

Leptospira are aerobic and grow best at 28 to 30 C Various media have been described most of which consist of serum saline enriched with peptone or meat infusion base in 0 2 per cent agar Chang (1947) recommends the addition of a small amount of an emulsion of fresh guinea pig liver to maintain virulence Leptospira will also grow in simple media consisting of salts thiamine asparagine and rabbit serum albumin (Schneiderman et al 1953) Growth is also obtained on the chorioallantoic membrane of the chick embryo (Morrow et al 1938 Chabaud 1939)

Species of Leptospira

In Table 50 are shown the principal antigenic types of leptospira together with certain related historical and epidemiologic data (Wolff 1953 Schlossberger and Brandis 1954) but the classification given is far from definitive Wolff (1953) for instance gives somewhat different relationships in a far more complicated scheme Expanding knowledge doubtless will enlarge both the geographic and the natural host range of these species All the pathogenic species seem to be distinct from the nonpathogenic *L biflexa* found the world over in small streams lakes and stagnant water but even these two groups have certain antigens in common The differentiation of the pathogenic strains is complicated by the marked degree of cross reactivity indicative of a considerable overlapping in antigenic structure and necessitating quantitative serologic tests and in particular careful antibody absorption studies Fractionation studies indicate that a somatic antigen probably a lipopolysaccharide is genus specific while a surface antigen is species specific The latter as yet chemically undefined may be recovered from culture supernates as well as from the organisms themselves (Rothstein and Hiatt 1956)

Therefore the division of this group of organisms into species and subspecies is made on a rather arbitrary basis and the studies of Bessemans and his associates (1948) in which exposure to specific antisera led to antigenic variants suggests that similar antigenic instability might occur in nature Moreover as knowledge of the leptospira has increased more caution has been necessary in identifying a single syndrome

Antibody studies have been carried out principally with agglutination lysis and complement fixation technics using whole cultured organisms as antigens Soluble antigens have also been used in complement fixation and precipitin tests The agglutination lysis test using living organisms as antigen is the test most frequently used although organisms killed by heat or formalin have also been employed It is unfortunate that more definitive studies on killed antigens or antigenic fractions have not been made since the lack of ready availability of living cultures of various species of leptospira has greatly limited the use of tests for leptospiral antibody in clinical and epidemiologic practice Other technics have been proposed but their uses and limitations have not been clearly defined Among these are the complement fixation test employing sonically disrupted leptospiral antigens (Randall Wetmore and Warner 1949 Schneider, 1954) hemagglutination of human O or sheep erythrocytes following sensitization with extracts of leptospira (Chang and McComb 1954 Cox 1955) and the use of infrared spectrophotometry for the differentiation of leptospiral species (Schneider and McLaughlin 1955) Endotoxins and exotoxins are believed not to occur although Alexander and his associates (1956) have recently demonstrated a soluble thermolabile oxygen stable hemolysin in certain species

Host Range and Pathogenesis

It is probable that wild rodents constitute the natural reservoir of all leptospiral species although some species have not yet been recovered from these animals In the United States several studies have shown that from 40 to 60 per cent of wild rats are naturally infected with leptospira (Li and Davis 1952) While in most of these studies the infecting organism has been considered to be *L icterohaemorrhagiae* only limited studies have been made to ascertain whether this is the only species of leptospira commonly carried by rats As indicated in Table 50, several species of leptospira have been recovered from mice field rats and other small rodents

Of the common experimental animal the guinea pig and the Syrian hamster are the most susceptible the young of each species being much more susceptible than the adult animal Dogs calves and pigs have been found naturally infected with several species and have also been infected experimentally Some

species notably *L icterohaemorrhagiae* appear to produce a more severe disease in guinea pigs and hamsters than others but it is not certain that this constitutes a reliable differential point for some strains of all species induce a mild and largely symptomless infection in these animals while an occasional strain of many species induces a more severe infection

Packchanian (1940) found 26 species and subspecies of American rodents to be susceptible to *L icterohaemorrhagiae* infection death occurring in 3 to 13 days usually preceded by jaundice Following intraperitoneal intramuscular or subcutaneous inoculation of infective material young (3 to 6 weeks old) guinea pigs and hamsters develop fever and loss of weight within 3 to 5 days followed by jaundice and multiple hemorrhages in the skin the subcutaneous tissue and the muscles Leptospira can be demonstrated readily in the blood the peritoneal fluid or the homogenates of the liver or the spleen In mild infections recovery is rapid and leptospira can only be recovered early in the infection

Wild rats and other small rodents cannot be used experimentally because of the likelihood of natural infection and even laboratory stocks have been found to be infected (Wolff et al 1949) Infection is rarely fatal in these animals and it is probable that they remain infected for life There is frequently an extensive and long lasting kidney involvement which can be demonstrated readily by darkfield examination culture or guinea pig inoculation and the animal may excrete leptospira in the urine during the rest of its life Water and food contaminated by the urine of infected rodents appear to be the principal means of spread to other animals and man

It is not known whether the initial site of multiplication of leptospira in infected animals and man is the blood or one or more of the internal organs but early in the disease leptospira can be recovered from the blood often the urine and from the liver the spleen the muscles and the serous surfaces including the meninges and the cerebrospinal fluid The liver and the spleen enlarge and an obstructive type of jaundice develops Petechial hemorrhages in the muscles the skin the sclerae etc are among the most common manifestations of the disease in both man and animals (Stavitsky 1945)

Leptospirosis in Man

Various clinical syndromes have become associated in the literature with a particular species of leptospira but as time goes on these syndromes appear to be less distinctive of any one species a possible exception may be pretibial fever Regardless of the species of leptospira involved the disease in man is characterized by an acute febrile illness muscle pains and headache albuminuria and the occurrence of multiple small hemorrhages which may be particularly noticeable in the sclerae In the classic Weil s disease originally believed to be due only to *L icterohaemorrhagiae* jaundice was a prominent sign but it is now known that many individuals infected with this species of leptospira do not have jaundice while some patients infected with the other types do present this sign In general perhaps *L icterohaemorrhagiae* and *L canicola* tend to give rise to a more severe type of human disease than do the other species although undoubtedly many mild cases of all types go undiagnosed evidenced by the occurrence of high titer agglutinins in individuals who have had no illness especially suggestive of leptospirosis (Ward and Turner 1942 Packchanian and Tom 1943) There is probably some degree of meningeal involvement in most cases and a not insignificant proportion of cases of aseptic meningitis have been found to be due to leptospirosis

Even in cases which can reasonably be attributed to a single species the disease in man has all degrees of severity varying from infections so mild as hardly to call themselves to the attention of the patient and recognizable only by serologic test to serious sometimes fatal illnesses with deep jaundice and profound prostration It usually begins after an incubation period of 6 to 15 days as an acute febrile illness which runs an irregular course some patients relapsing after an afebrile interval Conjunctivitis (episcleral injection) is a prominent and almost pathognomonic symptom The central nervous system may be involved with the appearance of the organism in the cerebrospinal fluid Meningeal involvement is particularly common in infections with *L icterohaemorrhagiae L grippotyphosa L canicola* and *L pomona* and several outbreaks of leptospiral meningitis have been described (Gauld et al 1952 Beeson and Hankey 1952) It seems clear that not

occur at the ends of the axis, but it is not known whether these have any function in the reproductive process it is believed that division is usually by transverse fission

Leptospira are aerobic and grow best at 28 to 30° C Various media have been described most of which consist of serum saline enriched with peptone or meat infusion base in 0 2 per cent agar Chang (1947) recommends the addition of a small amount of an emulsion of fresh guinea pig liver to maintain virulence Leptospira will also grow in simple media consisting of salts thiamine asparagine and rabbit serum albumin (Schneiderman et al 1953) Growth is also obtained on the chorio allantoic membrane of the chick embryo (Morrow et al 1938 Chabaud 1939)

Species of Leptospira

In Table 50 are shown the principal antigenic types of leptospira together with certain related historical and epidemiologic data (Wolff 1953 Schlossberger and Brandis 1954) but the classification given is far from definitive Wolff (1953) for instance gives somewhat different relationships in a far more complicated scheme Expanding knowledge doubtless will enlarge both the geographic and the natural host range of these species All the pathogenic species seem to be distinct from the nonpathogenic *L biflexa* found the world over in small streams lakes and stagnant water but even these two groups have certain antigens in common The differentiation of the pathogenic strains is complicated by the marked degree of cross reactivity indicative of a considerable overlapping in antigenic structure and necessitating quantitative serologic tests and in particular careful antibody absorption studies Fractionation studies indicate that a somatic antigen probably a lipopolysaccharide is genus specific while a surface antigen is species specific The latter as yet chemically undefined may be recovered from culture supernates as well as from the organisms themselves (Rothstein and Hiatt 1956)

Therefore the division of this group of organisms into species and subspecies is made on a rather arbitrary basis and the studies of Bessemans and his associates (1948), in which exposure to specific antisera led to antigenic variants suggests that similar antigenic instability might occur in nature Moreover as knowledge of the leptospira has increased more caution has been necessary in identifying a single syndrome

Antibody studies have been carried out principally with agglutination lysis and complement fixation technics using whole cultured organisms as antigens Soluble antigens have also been used in complement fixation and precipitin tests The agglutination lysis test using living organisms as antigen, is the test most frequently used although organisms killed by heat or formalin have also been employed It is unfortunate that more definitive studies on killed antigens or antigenic fractions have not been made since the lack of ready availability of living cultures of various species of leptospira has greatly limited the use of tests for leptospiral antibody in clinical and epidemiologic practice Other technics have been proposed but their uses and limitations have not been clearly defined Among these are the complement fixation test employing sonically disrupted leptospiral antigens (Randall Wetmore and Warner 1949 Schneider 1954) hemagglutination of human O or sheep erythrocytes following sensitization with extracts of leptospira (Chang and McComb 1954 Cox 1955) and the use of infrared spectrophotometry for the differentiation of leptospiral species (Schneider and McLaughlin 1955) Endotoxins and exotoxins are believed not to occur although Alexander and his associates (1956) have recently demonstrated a soluble thermolabile oxygen stable hemolysin in certain species

Host Range and Pathogenesis

It is probable that wild rodents constitute the natural reservoir of all leptospiral species although some species have not yet been recovered from these animals In the United States several studies have shown that from 40 to 60 per cent of wild rats are naturally infected with leptospira (Li and Davis 1952) While in most of these studies the infecting organism has been considered to be *L ictero haemorrhagiae* only limited studies have been made to ascertain whether this is the only species of leptospira commonly carried by rats As indicated in Table 50 several species of leptospira have been recovered from mice field rats and other small rodents

Of the common experimental animal the guinea pig and the Syrian hamster are the most susceptible the young of each species being much more susceptible than the adult animal Dogs calves and pigs have been found naturally infected with several species and have also been infected experimentally Some

culture (Alston and Broom 1944 Chang 1946) but the drug is not leptospiricidal even in large amounts The tetracyclines also inhibit growth in vitro in rather small amounts (0 2 to 20 gamma per ml) *L autumnalis* required considerably larger amounts of chloramphenicol to inhibit growth but it is not known whether or not there is a true species difference (Schlipkoter 1951) Leptospiricidal levels of tetracyclines are far higher than those clinically attainable in man (500 to 700 gamma/ml) In the experimental animal penicillin the tetracyclines and streptomycin are efficacious only when given soon after infection but have no effect if given when the experimental disease is well advanced

Numerous reports in the literature suggest that one or another antibiotic is effective therapeutically in man but the controlled series of 79 patients reported by Smadel (1953) failed to reveal a dramatic response On the basis of both clinical and laboratory evidence the preferred drugs are the tetracyclines in doses of 2 to 3 grams per day until the patient has been asymptomatic for several days

Epidemiology and Preventive Measures

Leptospirosis is primarily a zoonosis and man is an aberrant host Wild rodents appear to be the principal reservoirs it is not clear whether certain domestic animals known to be frequently infected with leptospira should be regarded as primary or secondary reservoirs The organisms are communicated to man either directly or indirectly by way of water or soil although leptospira do not survive long in nature outside an animal host The organism presumably enters the body through small breaks in the skin or through intact mucous membranes particularly the conjunctiva (Stavitsky 1945)

Many more animal species can be infected experimentally than have been found infected in nature For example leptospira have been transmitted experimentally to ticks (*O moubata*) which then became capable of infecting guinea pigs although neither of these species has been found infected in nature

In a given area one animal species generally harbors predominantly one serotype of leptospira or at most only a few serotypes which are weakly differentiated (Gsell 1953) Thus *L icterohaemorrhagiae* is found all over the world in the common brown rat and in some areas in other species of rats but rarely in other rodent species *L canicola* has been found principally in dogs but rarely in rats *L grippotyphosa* and *L hebdomadis* are found almost exclusively in the field mouse and the hamster *L pomona* has been found principally in pigs with perhaps secondary infection of cattle but rarely in rodent species it is possible that the antigenic specificity of the leptospira may depend largely upon components derived from host protein In general leptospiral infection in rodents is symptomless but experience in the laboratory with hamsters suggests that the young of the species may be more susceptible

Leptospiral infection of domestic animals has become associated with certain serotypes but the more infection i recognized the more exceptions to the general rule are discovered Thus infections in dogs usually have been with *L. canicola* or *L icterohaemorrhagiae* but most other species have been recovered occasionally cattle and horses also have been found infected with almost all species of leptospira

In man the similarity of the clinical syndromes caused by different species of leptopira has been referred to already and it is probable also that epidemiologic syndromes are no more specific but merely reflect opportunities for infection The commonest epidemiologic pattern is a situation in which man comes into frequent contact with water contaminated by leptospira containing urine of infected rodents or domestic animals The opportunities for this occurring are countless and the wonder is that human beings are not infected more often Thus the risk is comparatively great among sewer and mine workers soldiers in trench warfare bathers in swimming holes and canals workers in cane and rice fields and poultry dressers It has been shown for example that the sudden outbreak of human cases in the rice fields of Northern Italy corresponds to the invasion of these fields by mice early in July During this period great numbers of young mice are born so that the surface water is contaminated with urine containing leptospira (Mino cited by Gsell 1953) Likewise Kathe (1951) demonstrated that epidemics of swamp fever occurred in Silesia only when (1) abundant rain

only the severity of the disease but also its manifestations may vary widely in different areas and in different outbreaks and that the care used in looking for the disease affects the number of relatively mild cases discovered The febrile illness subsides by lysis after 3 to 10 days and may be followed by a second bout of fever In fatal cases there are hemorrhagic lesions in the kidney, the liver, the skin the muscles or the central nervous system The mortality is extremely variable ranging from 4 to 50 per cent in different outbreaks with an average of 5 to 10 per cent

Diagnosis

Definitive diagnosis rests on demonstration of leptospira or the appearance of specific antibodies in the serum Early in the disease the organisms may be demonstrated in the blood by animal inoculation or by growth in culture the use of both methods concurrently yields a greater number of positive results than either method alone Young guinea pigs or hamsters are inoculated intraperitoneally with 0 5 ml of citrated whole blood or blood plasma after light centrifugation the weight of the animal is taken daily and when the animal ceases to gain or loses weight it is sacrificed and peritoneal fluid, blood or suspensions of liver are examined under the dark field for leptospira Subculture of the test animals blood will also yield a few positive isolations that otherwise would be missed Direct examination of the patients blood is unreliable because of the difficulty of differentiating leptospira from red cell strands

The proportion of patients with demonstrable leptospira in the blood declines sharply during the second and the third weeks of illness but at this time the organisms may be recovered from the urine It is necessary to alkalinize the urine since leptospira rapidly disintegrate in an acid medium

Antibodies to the specific type of leptospira appear from the 7th to the 14th day of disease and rise sharply, as demonstrated by the agglutination lysis or the complement fixation test the former test is regarded as the more reliable A significant rise in antibody is particularly convincing Cross reactions between species of leptospira occur but it is desirable to use antigens prepared from several species In the United States antigens prepared from *L icterohaemorrhagiae L pomona* and *L autumnalis* should be used for ordinary diagnostic studies there is usually considerable serologic crossing between *L icterohaemorrhagiae* and *L canicola*

Immunity

The decrease in the number of organisms after the first 7 to 10 days probably reflects the development of specific antibodies The serum of convalescent patients protects guinea pigs and mice against an otherwise fatal inoculum and will cause lysis of the leptospira both in vitro and in vivo Agglutinating antibodies have been found to persist in the blood of recovered patients for many years Hyperimmune rabbit antiserum may have agglutination titers of 1 10 000 000 and 0 3 ml of such serum administered even 72 hours after inoculation may cure the infection in white mice As little as 0 003 ml prevents infection if given prophylactically at the time of inoculation (Larson 1943)

Artificial immunization has been carried out in animals and in human beings in a few areas Reactions in man tend to be severe and on the whole active immunization is not recommended However Wani (1933) claimed reduction in the incidence of Weil s disease following vaccination of coal miners in Japan with killed *L icterohaemorrhagiae* and numerous workers have demonstrated satisfactory rises in leptospiral antibodies following vaccination with heat killed organisms (Esseveld 1937 Walch Sorgdrager 1939 Schuffner 1941 Borg Petersen 1953) Dogs have been immunized successfully with killed cultures by Dalling and O Kell (1926) and more recently experimental animals including dogs have been successfully immunized with both heat killed and formalin killed organisms (Brunner and Meyer 1950 Broom 1951 Borg Peterson 1953)

Treatment

No specific treatment yields dramatically effective results Specific antiserum therapy and the use of the sulfonamides have been discarded while favorable results with one or another antibiotic have been reported the results of carefully controlled experiments have usually been disappointing

Penicillin in amounts as small as 0 4 units per ml (about 0 25 gamma per ml) will inhibit the growth of *L icterohaemorrhagiae* in

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The Bartonella Group

This group consists of one human pathogen, *Bartonella bacilliformis*, the cause of Carrion's disease, and of micro-organisms of similar appearance occurring in other animals and classified in the genera *Haemobartonella* and *Eperythrozoon*. All infect erythrocytes and usually cause primary infectious febrile anemias. *Bartonella bacilliformis* is an undoubted bacterium. At present all these organisms are classified in the family Bartonellaceae, order Rickettsiales, in the 7th edition of Bergey's *Manual of Determinative Bacteriology*.

BARTONELLA BACILLIFORMIS

Definition

Bartonella bacilliformis produces unique and striking clinical conditions which serve better to define the organism than the rather undistinguished characteristics thus far discovered in vitro. In addition to nonclinical asymptomatic infections, *Bartonella bacilliformis* causes two very different and apparently unrelated conditions: (1) Oroya fever, a febrile anemia, and (2) Verruga peruana, a benign skin eruption. Each form has a distinctive pathologic substratum, and the two are linked immunologically. In Oroya fever *Bartonella* is found on the erythrocytes. In both diseases it occurs within fixed tissue cells, notably those of the reticuloendothelial system. On the red cells it is unmistakable and no other human parasite resembles it even slightly (Fig. 55-1 and 2). In the tissues it is intracellular and during Oroya fever multiplies within the cytoplasm of vascular endothelial cells, grouped in rounded masses or as isolated elements (Fig. 55-3). It can be cultivated by unlimited serial transfers on cell-free media.

GENERAL CHARACTERISTICS AND HISTORY OF BARTONELLOSIS

Bartonellosis is a microbiologist's curio since it is unique not only in the duality of its clinical forms, a duality expressed too in the pathology of each, but it is also distinctive in the interrelation and the opposition between the two forms. These latter characteristics reflect in all probability successive immunologic states of the same individual. It is one of the most perfect examples of a geographically restricted bacteriologic infection, being exclusively American, exclusively tropical, exclusively Andean, and proved to exist only in mountainous regions of Peru, Colombia, and Ecuador.

Oroya fever is a febrile hemolytic macrocytic anemia of rapid evolution and, if untreated, of high mortality. *Bartonella bacilliformis* is readily visible in blood films, becomes progressively more abundant in the blood with the aggravation of the anemia, and may infect over 90 per cent of the erythrocytes. The mortality rate of untreated Oroya fever is about 40 per cent. If the patient recovers, he often develops the second form of the infection, verruga peruana. This is marked by a generalized eruption of intensely colored red-purple vascular papules interspersed with more deep-seated nodular elements and is usually benign, with a death rate below 5 per cent.

accompanied an epizootic in field mice (2) agricultural areas were covered by flood water, and (3) the mean daily temperature was not lower than 18° to 19° C

On the other hand domestic animals can account for outbreaks of leptospirosis in man independent of conditions of the climate and the soil Infection may occur through contact with the infected animals urine or by handling the flesh of diseased animals Bemkopf (1947) has shown that refrigerated meat of bovine carriers may remain infective for 33 hours after slaughtering The incidence of leptospirosis in man may change in any region according to changing conditions of animal husbandry

While leptospirosis can occur in all ages at all seasons and in both sexes for the reasons given above it is primarily a disease of young male adults of warm weather and of certain occupational groups Preventive measures vary according to (1) the ecology of the carrier host (2) the mode of transmission of infection and (3) the population at risk and they must be developed to meet the different epidemiologic patterns encountered Elimination of rat infestation in eating establishments and homes will prevent a small proportion of cases Infections from bathing in official pools can be prevented by denying access to rats through proper sanitation and screening (Walch Sorgdrager 1939) Little can be done to reduce the risk of bathing in natural streams and stagnant pools except to warn individuals of the risk (Gauld et al 1952) The risk of infection among sewer workers and swine herds is greatest in those who go barefoot wearing strong boots is therefore a preventive measure Leptospirosis has been controlled among poultry dressers and fishmongers by correction of unsanitary conditions in which offal rats and standing water provided opportunities for the spread of infection (Davidson et al 1934 Ward et al 1942) A marked reduction in the incidence of canefield fever has been obtained by burning the dry leaves of standing cane which grows in low lying areas Burning is done just before cutting and does not damage the canes it drives out rats and evaporates rat urine *and small pools of* surface water (Broom 1953) Protection of the hands by heavy gloves has been recommended for butchers and other workers who handle potentially infected meat

Of the three links in the chain of transmission—the carrier, the leptospira outside the body of the carrier and the susceptible host—preventive measures have been directed most often against the center link by ratproofing or by the wearing of protective clothing No effective vaccine is available for use in man or domestic animals

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even when untreated Verruga persists with successive crops for from a month to a year but unlike Oroya fever, anemia if present is moderate in degree and during all of this period *Bartonella* will not be seen in blood films although it can be cultivated from the blood

Verruga was known to the pre Columbian Indians whereas the anemic form did not attract much attention until 1870 when an epidemic caused an estimated 7 000 deaths Carrion s experiment in 1885 in which he was inoculated with verruga material and died of Oroya fever 39 days later strongly suggested the etiologic unity of the two conditions The micro organism was reported by Barton in 1909 Its relationship to Oroya fever was confirmed in 1915 by Strong Tyzzer Brues Sellards and Gastiaburu who also described the pathology of Oroya fever and discovered the pathognomonic lesion found therein Noguchi and Battistini in 1926 cultivated the organism described the relation of *Bartonella bacilliformis* to verruga thus producing experimental evidence supporting the unitarian etiology and Noguchi subsequently gave detailed descriptions of the cultural and biologic characteristics of the cultivated organism These results not duplicated at first in other laboratories were finally fully confirmed by the Harvard 1937 Expedition to Peru (Weinman and Pinkerton Pinkerton and Weinman) which also noted the prevalence of nonclinical or latent infections in man Taken in conjunction with the prior discovery of phlebotomus as the vector by Shannon and by Noguchi this has given a much clearer picture of the conditions for perpetuation and extension of the disease Hertig (1938) re identified the species of phlebotomus concerned and with Fisher (1945) indicated that control might be achieved with DDT a result which has since been accomplished in a number of areas The major therapeutic problem i e the treatment of Oroya fever, appears to have been solved by the use of antibiotics A monograph on the subject published in 1944 (Weinman) treats of the history in great detail subsequent references will be found in previous editions of this volume

MORPHOLOGY AND BIOLOGIC PROPERTIES OF BARTONELLA

Bartonella bacilliformis is a gram negative organism which stains unsatisfactorily with the customary bacteriologic stains but a distinct red violet with Wright s or Giemsa s fluid It is very polymorphous Flagella numbering 1 to 10 and always unipolar have been demonstrated on culture material (Fig 56) but never satisfactorily on the blood forms otherwise the maximum morphologic range is seen in the blood of man where rings commas disks and rods long short and arranged in chains may all be seen (Fig 55)

The culture medium most commonly used

FIG 55 All blood films were stained with Giemsa s fluid after fixation either by the May Grunwald mixture or by methyl alcohol (Weinman D 1944 Infectious anemias due to bartonella and related red cell parasites Tr Am Philosophical Soc *33* 243 350)

(1 and 2) *Bartonella bacilliformis* in blood films of two fatal cases of Oroya fever In Figure 1 the infection is intense the bartonellae parasitize not only the erythrocytes but are also found within monocytes (Original)

(2) The straight and curved rods chains dots and rings illustrate the great morphologic range of *B bacilliformis* (Original magnification as in 1)

(3) Section of an Oroya fever lymph node Development of *B bacilliformis* in distended endothelial cells lining the vein The intracellular distribution in rounded clumps i distinct in the heavily infected cells (Redrawn from C Uribe)

(4) Section of a human verruga Regaud fixation Giemsa stain The blood capillaries are numerous and the proliferated endothelial cells conspicuous *B bacilliformis* stains red is very evident and is often located distinctly within the cytoplasm of endothelial cells Note that despite the numbers of parasites in the ti sues none appear upon the erythrocytes (Original)

(5) *Haemobartonella muris* in blood films of splenectomized mou e Variation in shape is extreme Rods coccoids and rings occur both singly and in various combinations constituting bow forms filaments which contain rings or coccoids rows of rings and coccoids etc (Redrawn from F E Tyzzer)

(6) *Eperythrozoon coccoides* in the blood of a splenectomized white mou e An intense infection in which as is customary rings preponderate The resemblance to certain forms of *B bacilliformis* is quite noticeable (Original)

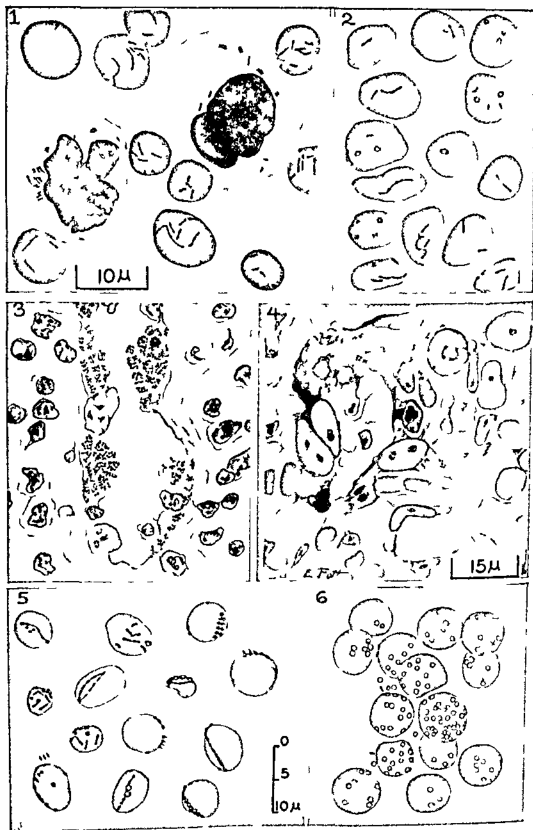


FIGURE 55 (*Caption on facing page*)

inocula such as cultures verrugas of either human or simian origin or tissue from Oroya fever patients injected into the skin or subcutaneous tissue of *M mulatta* produce a local verruga which is sometimes multiple but very rarely generalized Oroya fever blood almost never produces verrugas in monkeys yet infected lymph nodes from an Oroya fever patient or cultures made from Oroya fever blood will produce verrugas in almost every instance when appropriately injected This is a puzzling feature and is not explicable by quantitative factors alone

PATHOLOGY AND PATHOGENESIS

In Oroya fever the body is pale and somewhat icteric the liver enlarged the spleen frequently so and often infarcted and the lymph nodes usually enlarged are often hemorrhagic

The microscopic appearance is distinctive and results from the development of masses of barotonella within the cytoplasm of cells lining the blood and the lymph capillaries The growth reaches an extreme degree causes the cytoplasm to expand to many times its normal width and to bulge into the lumen of the vessel The entire cytoplasm may be filled with micro organisms which tend to form rounded masses or clumps (Fig 55 3) Infected cells are particularly abundant in lymph nodes and also occur in the liver the spleen bone marrow the kidney the adrenals the pancreas more rarely in the skin the heart and the lung Gross lesions such as the splenic infarcts may follow upon vascular occlusion by the swollen endothelium

Verruga peruana differs as much in microscopic appearance from Oroya fever as do the clinical entities It is a specific granuloma with three characteristic features It is very vascular as a result of the formation within the element of numerous small caliber blood vessels Proliferating endothelial cells are present in abundance they frequently occur in masses or islands and lie in edematous connective tissue the whole being infiltrated with a variable number of lymphocytes plasma cells and polymorphonuclears Lastly *Bartonella* is present in the lesion usually within the cytoplasm of the tissue endothelial cells or in their neighborhood Swollen vascular endothelial cells bulging into the lumen of the vessel which is such a feature of Oroya fever, are almost totally lacking (Fig 55 4)

The anemia of Oroya fever is due primarily to blood destruction but the intimate mechanism of the hemolysis has resisted analysis There is no evidence for the existence of a lysin of any sort in vivo and *Bartonella* is not hemolytic in vitro For verruga there is at present no information correlating the complex cellular and tissue reactions with the properties or constituents of *Bartonella*

The great variability in response to *Bartonella bacilliformis* infection ranging from Oroya fever to verruga not preceded by Oroya and finally to nonclinical latent infection probably is due to variations in both the host and the micro-organism Controlled experiments in animals are available with regard to verruga and asymptomatic infection and do indicate a great range of host susceptibility and of pathogenicity of the organism In Oroya fever both factors probably vary also but since the condition is not reproducible at will in animals the matter is not certain No differences have been detected between bartonellae isolated from Oroya fever cases and those obtained from verruga patients They are similar in pathogenicity give various cross immunity tests in animals have the same morphology and behave similarly in vitro Both flagellate and aflagellate strains are pathogenic

IMMUNITY

The fundamental concept is that infection is long continuing and exhibits two successive clinical manifestations of which the second verruga coincides with and seems to be an actual expression of immunity toward the first form Oroya fever If a patient has undergone Oroya fever then has had verruga from which he has recovered usually he will not have a second attack even though exposed But should the second attack occur it will almost invariably be verruga and not Oroya fever This immunity to second attacks does not always signify that the infection has been eradicated On the contrary *Bartonella* can be cultivated from the blood of recovered patients for long periods of time This latent or asymptomatic infection is a characteristic feature of the bartonelloses animal as well as human Latent infection is not limited to postconva

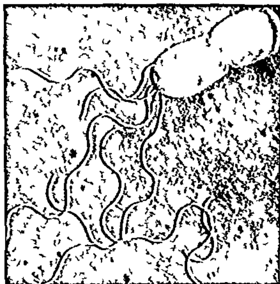


FIG 56 *Bartonella bacilliformis* from an 8 day culture Hemolyzed palladium shadowed Note unipolar flagella cell membrane and commencement of binary division Magnification × 19 000 (Peters D and Wigand R 1952 Neue Untersuchungen uber Bartonella bacilliformis I Morphologie der Kulturform Ztschr Tropenmed u Parasitol *3* 313 326)

is Noguchi s leptospira medium a semisolid nutrient agar containing 10 per cent rabbit serum and a small amount of rabbit hemoglobin (0 50% or less) Growth becomes evident in this medium in from a week to 10 days at 28° C sometimes longer in a band about 1 cm wide and about 5 mm below the surface Colonies if minute produce only a faint haze but they may grow to be visible white spherical bodies from 1 to 2 mm in diameter The organisms occur singly and in large and small irregular dense collections with jagged edges 25 to 50 μ or more in diameter Rod forms predominate in young (10 to 20-day) cultures and granules measuring as little as 0 2 x 0 3 μ in older ones Flagella are demonstrable on motile strains by silver impregnation methods and are beautifully shown by the electron microscope after palladium shadowing (Fig 56) The flagella are unipolar arise from the cytoplasm are 1 to 10 in number vary in length from 3 to 10 μ and have a diameter of about 20 μμ and undulation phases of 0 95 μ (Noguchi, Peters and Wigand 1952) As noted above flagella have not been demonstrated with certainty on the epierythrocytic forms, and aflagellar strains exist A definite cell membrane can be demonstrated by appropriate technics applied to either blood or culture forms (Fig 56) In this respect *Bartonella* resembles other gram negative bacteria but differs from apparently closely related members of the Bartonella group (Peters and Wigand, 1955) *Bartonella* does not traverse the usual bacteriologic filters (e g Berkefeld N)

The most favorable temperature for the semisolid cultures is 28 C The optimum pH is 7 8 with a range from 6 8 to 8 4 *Bartonella* is an obligate aerobe It produces neither acid nor gas with any of the numerous carbohydrates tested does not liquefy gelatin and has no action on lead acetate All attempts to isolate a hemolysin have failed it does not hemolyze red cells in culture or even localize on or about the erythrocytes when they are added to a semisolid medium Pathogenicity is retained in semisolid leptospira medium for several years at least whereas blood agar slants are generally less favorable in this respect Only media containing blood have been successful serum or plasma has appeared to be essential and hemoglobin is favorable because it supplies hemin Enrichment of media with glutathione and ascorbic acid may give heavier growth according to Geiman *Bartonella* is sensitive in vivo to penicillin streptomycin chloramphenicol and tetracycline or ganic arsenicals are relatively inactive It is a facultative intracellular parasite when grown in tissue culture and then reproduces the appearance seen in necropsy or biopsy material

RANGE OF PATHOGENICITY

Bartonella manifests full pathogenicity for man only In certain monkeys and apes notably *Macaca mulatta* (*M rhesus*) verrugas may be produced with great regularity and passed in series but Oroya fever cannot be reproduced in animals at will and splenectomy has not made it possible to produce predictable Oroya fever infections in monkeys (Noguchi Weinman and Pinkerton Wigand and Weyer 1953) Usual laboratory animals, including the developing chick embryo are not satisfactory even for verruga production The route of inoculation and the nature of the inoculum are of prime importance in determining the character of the infection Satisfactory

EPIDEMIOLOGY

Bartonella is transmitted by one or probably several species of *Phlebotomus* or sand fly indigenous to the endemic regions. There are records of a few cases of congenital infection, but bartonellosis is not transmitted by ordinary contact. The sources of the microorganism are limited almost exclusively to the two hosts, man and phlebotomus, whence the importance of the asymptomatic infections. The 5 to 10 per cent of the apparently healthy population in endemic areas which have *Bartonella* circulating in the blood, as well as postconvalescent carriers and patients, serve as possible sources of infection to phlebotomi. The fact that repeated cultures were obtained only rarely from infected humans has been cited as evidence against the importance of the human reservoir (Herrer 1953b). This fact may be interpreted equally well as evidence of the inefficacy of the cultural method as compared with the efficiency of *Phlebotomus* in favoring growth. Meanwhile, no important source of *Bartonella* other than man has been discovered (Herrer 1953a).

Once implanted in a region, bartonellosis tends to remain almost indefinitely; in a focus reported in 1630 the infection has persisted for over 300 years. Epidemics arise through infection of a previously nonexposed and therefore nonimmune population. Such a population may be introduced within an endemic area to produce an internal epidemic not correlated with geographic extension (e.g., the 1870 Peruvian outbreak), or there may be an actual invasion of new territory. This seems to have been the case in Colombia in 1938, where bartonellosis caused an estimated 4 000 deaths.

Bartonellosis is contracted only in northwestern South America, exclusively in mountainous areas, solely in the neighborhood of river valleys where the elevation must be neither too high (below 8 000 feet) nor too low (above 2 500 feet), and usually only at night. The explanation lies in the biology of the vector. The pertinent facts are available for *P. verrucarum* in Peru. This phlebotomus is restricted to certain areas by requirements of moisture and temperature. Above a certain altitude the night temperature is too low; below a certain limit the rainfall is insufficient and conditions are too arid. Transmission occurs only at night because the phlebotomi feed only then. Phlebotomi are the only natural vectors known, and *P. verrucarum* the only species for which transmission is proved. Very probably others are involved. In Colombia, where the Peruvian species have not yet been found, the epidemic apparently was not caused by the importation of infected sand flies but by the infection of native phlebotomi, possibly by human carriers; the presumptive vector is *P. columbianus* (Rozeboom 1947). Experiments involving *Pediculus humanus* have not supported the view that lice might spread the disease (Wigand and Weyer 1953).

CONTROL MEASURES

DDT appears to have furnished a simple, relatively inexpensive method for community sanitation. The application of residue sprays to the inside of residences and sleeping quarters, to the outside of doors and windows, and to likely breeding spots in the immediate vicinity was shown by Hertig and Fisher to exert effective phlebotomus control. In Peru, in an endemic region, Corradetti applied these technics and succeeded in protecting a group of about 900 men for several months, not only from bartonellosis but to a marked degree from malaria also, and this despite the frequent moving of the work camps as the construction project progressed. Thus far resistance to DDT by *Phlebotomus* has posed no problem.

Individuals may protect themselves by nightly removal from the phlebotomus zone, by the use of insect repellents and DDT as indicated, and presumably by the prophylactic use of active antibiotics. There is no vaccine of demonstrated efficacy.

HEMOBARTONELLA AND EPERYTHROZOON

The hemobartonellae resemble *Bartonella bacilliformis* in appearance but do not cause any condition akin to verruga, do not multiply massively, if at all, within vascular endothelial cells, and are widely distributed geographically. Their very different response to chemotherapy indicates fundamental metabolic differences; in any case they are unsuitable test organisms for anti-*Bartonella* activity. Because of the splenic immunity asso

lescence and persons with no history of the disease can be carriers From 5 to 10 per cent of the population in bartonella infected areas are carriers and probably play an important part in maintaining endemicity

Complement fixation has been reported with cultures as antigens strains of varying origins gave no significant titer differences in quantitative tests Agglutinins are present during both phases of the disease and in carriers It is doubtful that they play any important part in acquired immunity Limited attempts at immunization with formolized cultures induced agglutinin formation but did not prevent infection Perhaps one or more of the antibodies described above may inhibit the development of *Bartonella* in vitro for Herrer (1953b) found that cultures are more likely to be positive in the first months following infection than later even though infection continues throughout as was demonstrated by the eventual outcrop of verrugas

DIAGNOSIS

Oroya fever usually offers no diagnostic problem once the anemia is pronounced *Bartonellae* can then be found in stained films by blood examination Prior to the anemic period blood cultures can be positive the incentive to make them comes from knowledge of prior residence in an endemic zone and suggestive symptoms of the preanemic period unexplained fever and joint pains A generalized well developed verruga eruption has a very distinctive appearance Individual elements may resemble those of other eruptive conditions but the histology is usually very different Cultures from the verrugas are usually unsatisfactory due to contamination but cultures of *Bartonella* may be obtained from the blood throughout the course of the eruption The diagnosis of latent infection also depends upon blood cultures In all cases cultures if negative should be repeated for, particularly during the verruga period it may be very difficult to cultivate *Bartonella* from the blood (Herrer)

The identification of *Bartonella bacilliformis* is based upon the following points isolation from patients with a typical case of one of the two forms of the disease or from carriers with a history of residence in endemic areas or from infected sandflies characteristic growth in a semisolid medium pathogenicity for *Macaca mulatta*, in which verrugas may be produced the morphology and the cellular situation in vivo and the appearance staining and biochemical reactions in vitro *Some strains are nonpathogenic* and for these instead of the monkey test tissue cultures can be substituted growth will be both intracellular and extracellular and within cells the organism will show a tendency to grow in rounded masses

TREATMENT

Satisfactory clinical results can be obtained even though control of the infection is incomplete If a patient recovers from Oroya fever he may continue to yield positive blood cultures and to develop verrugas but does not die even though still infected presumably because he develops immunity

Oroya fever patients respond with dramatic improvement to penicillin (Merino Aldana and Tisnado) streptomycin (Aldana Gastelumendi and Dieguez Krumdieck) chloramphenicol (Payne and Urteaga Urteaga and Payne 1955) or tetracycline (Araujo 1955) Fever disappears usually in 48 hours or less the bartonellae change in morphology the rod forms being replaced by coccuslike types then (after several days if not before) they diminish in numbers while the blood count stabilizes and then increases Presumably the antibiotics control the infection and allow the immunologic mechanisms to come into force

Choice of antibiotic will be regulated by secondary considerations (Aldana Zubiate Contreras) As has been reported for some years secondary *Salmonella* infection of Oroya fever patients is not infrequent and is of poor prognosis Therapeutic agents with activity against both *Bartonella* and the salmonellae probably will come to be preferred and favorable reports on the action of chloramphenicol in such combined infections has already been reported (e g Urteaga and Payne 1955) For this reason chloramphenicol is favored over other antibiotics with anti Bartonella activity (Cuadra 1956) even though there is some in vitro evidence that it is not the most potent (Wigand 1952) Symptomatic relief of the anemia is accomplished by transfusions which it may be advantageous to repeat to total 3 to 8 liters (Hodgson)

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Streptobacillus Moniliformis

DEFINITION

Streptobacillus moniliformis is an aerobic gram negative pleomorphic micro organism requiring blood serum or ascitic fluid for cultivation In the animal body and in young cultures grown in completely satisfactory media the morphology is more uniformly bacillary (2 to 4 μ in length) whereas in older cultures and in cultures in slightly unsatisfactory media the morphologic forms consist of filaments up to 40 μ in length and chains of bacilli or cocci Often these filaments show swellings 2 to 5 times their own width The words *Streptobacillus* and *moniliformis* constitute the most succinct description of the morphology of this organism A variant designated L_1 (L = Lister Institute) is produced The organism is a normal inhabitant of the nasopharynx of wild and laboratory rats It is highly pathogenic for mice nonpathogenic for guinea pigs rabbits and usually rats In man it is the cause of one type of rat bite fever and of a disease known as Haverhill fever or erythema arthriticum epidemicum which is characterized by fever rash and polyarthritis but is not acquired by the bite of a rat (Synonyms *Streptothrix muris ratti Actinomyces muris Asterococcus muris Haverhillia multiformis* etc)

HISTORY

The fact that rat bite fever may be caused by two different micro organisms *Spirillum minus* and *Streptobacillus moniliformis* and the extreme variation of the latter organism have lead to a very confused literature The first case of rat bite fever was described by Wilcox in 1840 but the role of *Streptobacillus* under various names was not discovered until 1914 and the years immediately ensuing The epidemics in Chester Pa (Place and Sutton 1934) and in Haverhill Mass (Parker and Hudson 1926) have demonstrated that the bite of a rat is not essential for man s acquiring the infection For a good review of the disease and its causative organisms the reader is referred to the publication of Brown and Nunemaker (1942)

CULTIVATION AND BIOLOGIC PROPERTIES

One of the characteristics of *S moniliformis* is that it requires the presence of a natural body fluid for growth on artificial medium Tryptose phosphate broth and media of the infusion type are satisfactory basal media These require enriching with blood serum or ascitic fluid to a concentration of 10 to 30 per cent The organisms grow better in media with a pH of about 7 6 Optimum growth takes place in an aerobic atmosphere at 37° C Growth on solid medium is favored if the basal medium contains slightly less than the conventional 1 5 per cent agar and the atmosphere contains a high moisture content In a few instances it has been found possible to replace the serum component in the medium by starch (Heilman 1941b) glycogen or dextrin (Dumoff and Duffy 1951)

In fluid cultures growth usually takes place

ciated with them the group has attracted particular interest *Haemobartonella muris* found in wild and many strains of laboratory white rats provokes a fulminating anemia accompanied by hemoglobinuria and death A high percentage of rats are infected with *H muris* yet they show no signs of the dis ease may never do so and react mildly if at all to inoculation of the organism But if the spleen is removed from these rats, the para sites multiply and the anemia evolves in its acute form The infection is contracted at an early age and usually remains latent prob ably throughout life unless splenectomy is per formed Other procedures may have an effect similar to that of splenectomy i e certain asso ciated infections x ray irradiation α particle irradiation from injected Polonium 210 (Scott and Standard 1954) Quite possibly all of these act to impair the efficiency of the spleen Although *Haemobartonella* infections occur in a great variety of animals none is known in man the human postsplenectomy iron staining structures, Pappenheimer bodies probably not being living organisms The cus tomary effect of splenectomy on *Bartonella bacilliformis* infections is not known but Oroya fever following splenectomy has once been reported (Aldana and Urteaga 1947) *Eperythrozoon* has not been proved to occur in man although a possible human infection has been reported Splenic control is a promi nent feature of eperythrozoon infections and the mouse organism *E coccoides* (Fig 55 6) has again attracted attention because it is a striking example of a latent infection which converts an otherwise benign infection with the hepatitis virus into a fatal disease at the same time that it induces a many fold increase in virus titer (Niven et al , 1952)

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in the form of *fluff balls* or *bread crumbs* on the bottom of the tube or on the surface of the sedimented red blood cells The supernatant fluid may remain clear and devoid of organisms A *fluff ball* should be removed with a pipette for transfer or microscopic examination Giemsa s and Wayson s stains are more satisfactory than Gram s technic for demonstrating the micro-organisms Fluid cultures may require daily transfer in order to maintain the viability of the strain

Another characteristic of the organism is its extreme pleomorphism in microscopic preparations The cells vary from fairly uniform small slender rods to long slender filaments which are often irregularly fragmented and are said to resemble the dot dash appearance of the Morse code In addition they show circular to spindle shaped swellings (Fig 59)

On solid medium growth takes place in 2 to 3 days more slowly than in liquid media and the organisms are viable for a longer period i e up to 1 week Individual colonies are raised and granular and may become as large as 5 mm in diameter Beneath or adjacent to the colonies of *Streptobacillus* the microscopic L_1 colonies may develop Organisms in the L_1 colonies are much more resistant to penicillin than are those in the *Streptobacillus* colonies consequently penicillin can be used to isolate the L_1 component The L_1 organism can be obtained and maintained in pure culture and can be transformed back to the *Streptobacillus* organism Heilman 1941a) Compared with *S moniliformis* L_1 is less virulent if virulent at all for the mouse and it is a poorer immunizing antigen (Freundt 1956b) *S moniliformis* has been reported to produce acid without gas from glucose maltose levulose salicin starch and glycogen and to being inert toward lactose sucrose mannitol xylose inositol inulin dulcitol arabinose raffinose sorbitol trehalose rhamnose and glycerol It is variable toward galactose The L_1 variant has fermentative activities identical with those of *S moniliformis* (Heilman 1941b) An interesting association of two micro-organisms is the dependence of *Entamoeba histolytica* upon its associated *Streptobacillus* for metabolism of glucose (Loran et al 1956)

The view of Dienes (1939) is now generally accepted that the L_1 organism is a variant of *S moniliformis* An effort is being made (Brassermann et al , 1957) to employ the term L with some consistency It is recommended that this term be reserved for a type of growth arising spontaneously or by stimulation which is characterized by a colony of special appearance howing a dense center and lighter periphery on media not containing the original stimulating substances Further more micro-organisms compri ing this L form must not assume the morphology of the parent culture Organisms in the L_1 colonies grow into the agar medium so that they cannot be transferred by the usual bacteriologic technics using an inoculating needle The agar block transfer technics of Klieneberger (1935) give the best results

The organisms are extremely fragile and soft Young colonies and the central mass of well developed colonies consist of small forms which often appear as tiny bipolar stained bacilli These forms are tran formed at the surface and at the periphery of the colonies by gradual swelling into large round forms The morphology of the organi ms in the L_1 colonies is so similar to that of pleuropneumonialike organi ms that without knowing the origin of a culture after it has lost its ability to return to the *Streptobacillus* it would be impossible to differentiate between the two on the basis of morphology (Dienes 1947)

The production of L colonies from bacteria and the reversion to the bacillary phase has been interpreted by Klieneberger Nobel (1951) as a process of regeneration in bacteria initiated by a fusion of certain elements In some cases when the multiplication rate of bacteria is disturbed during a state of vigorous growth the bacteria react by transforming into the L phase in which the organisms can better withstand the adverse conditions Organisms in the L phase multiply by seg

Fig 58 (*Continued*) in situ with methylene blue) Note the irregular wavy filaments containing deeply stained pear shaped swellings Magnification × 1855

Fig 59 (*Bottom right*) Broth culture of *Streptobacillus moniliformis* (Gram stain of 24 hour growth) Note the lack of pleomorphism Magnification × 1855 (Dr Louis Dienes)





Fig 57 (*Top*) *Streptobacillus moniliformis* and pleuropneumonialike organism L (24 hour culture on ascitic fluid agar stained in situ with methylene blue Azure II) Magnification × 112 (Dr Cynthia H Pierce)

Fig 58 (*Bottom left*) *Streptobacillus moniliformis* colony (24 hour agar culture stained

the rat is regarded generally as being fairly resistant to infection by S moniliformis one recently isolated strain has been found to be pathogenic for the laboratory rat (Lerner and Silverstein 1957) The animals showed an osteoarthritis which was often accompanied by an osteomyelitis periostitis arthritis and periarthritis

S moniliformis is unusual in that it has been demonstrated to produce in vivo the L_1 variant which is endowed with properties of greater resistance and this variant is capable of reverting to *Streptobacillus*

DIAGNOSIS

Rat bite fever resulting from *S moniliformis* may vary in its symptomatology in individual cases but the clinical picture is sufficiently characteristic so as not to be overlooked readily (Blake 1916) A typical case presents usually but not always the history of a rat bite a latent incubation period of variable length with subsequent nonsuppurative inflammatory reaction at the site of the wound lymphangitis enlarged lymph nodes severe chill at onset high fever of the relapsing type leukocytosis intense muscular pains nervous symptoms and bluish red exanthem Endocarditis and arthritis may be complications and a false positive Wassermann reaction may occur

It is important to attempt to confirm the tentative clinical diagnosis of rat bite fever by demonstrating the causative organism This may be either *Spirillum minus* or *S moniliformis* *S minus* is demonstrated by animal inoculation whereas *S moniliformis* is demonstrated by cultural methods

S moniliformis may be isolated in routine blood cultures from patients if a good culture medium is employed such as infusion broth or tryptose phosphate broth and a sufficient amount of the patient s blood is added to each container of broth to give a concentration of nearly 20 per cent When growth occurs it is found on the surface of the sedimented blood cells and on the inclined surface of culture tubes and this growth resembles bread crumbs fluff balls or cotton balls in its gross appearance The organism has also been isolated from the fluid aspirated from involved joints

An agglutinin titer of 1 80 or greater with *Streptobacillus* antigen is considered as indicative of infection with that organism The specific *Streptobacillus* agglutinins may appear as early as 10 days or as late as 2 5 months after the bite, may attain their maximum titer in 1 to 3 months after the bite and may disappear within 5 months or persist for over 2 years (Brown and Nunemaker 1942)

TREATMENT

Penicillin inhibits the growth of *S moniliformis* in vitro protects mice against experimental infections (Heilman and Herrell 1944) and appears to be effective in the treatment of some cases of the natural infection in man (Altmeier et al 1945 Wheeler 1945) Streptomycin was found to be effective in the treatment of a natural infection which did not respond to penicillin (Sprecher and Copeland 1947) and to be more effective than penicillin in the treatment of mice after joint involvement had developed (Levey and Levey 1948) The L_1 variant along with pleuropneumonia like organisms is resistant to penicillin and although penicillin may free the body of the *S moniliformis* the L_1 organism may persist and a broad spectrum antibiotic such as chlortetracycline must be employed to bring about its elimination from the body (Dolman et al 1947)

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mentation they eventually break up into small granules which are capable of germination and in some cases are filterable In addition the L forms show differences in food requirements and in metabolism such as resistance to penicillin and other chemicals (Edward 1953)

EPIDEMIOLOGY

Infections with *S moniliformis* are usually acquired through the bite of a rodent (rat squirrel, weasel) Frequently, there is no known history of rodent bite or animal contact but there may be a history of trauma In rare instances the infection may be acquired from contaminated food

S moniliformis is a normal inhabitant of the respiratory tract of laboratory and wild rats Being present particularly in the upper portion of the respiratory tract the nasopharynx the organisms are transmitted readily during the act of biting by the animal In rats there may be involvement of the lungs and the middle ears but generally rats are *refractory to infection (Strangeways 1933)* Investigators have failed to isolate the organisms from the mouths of normal healthy mice but occasionally an epizootic disease resulting from *S moniliformis* occurs among laboratory (Freundt 1956a) and wild mice (Williams 1941) There is no reported case of rat bite fever in man as a result of the bite of a mouse but man has acquired the infection by handling mice (Levaditi et al 1925) Squirrels and weasels have been known to transmit the infection

Cases have been reported in which there is no history of a rodent bite or contact with animals such as that reported by Sprecher and Copeland (1947) In one instance the infection developed after a fall (Levey and Levey 1948) and in another instance it may have been associated with trauma (Hazard and Goodkin, *1932)*

Contaminated food may be the source of sporadic outbreaks or isolated cases The epidemics in Chester, Pa in 1925, and in Haverhill Mass, in 1926 are suspected of having been milk borne (Place and Sutton 1934) and the case reported by *Oeding and Pedersen* (1950) is also ascribed to contamination of food or milk

PATHOGENESIS

From the clinical features, the pathogenesis of rat bite fever becomes obvious Following the inoculation of a susceptible individual with *S moniliformis* through a bite wound, there is a proliferation of the organisms at the site of the wound, extension to the neighboring lymphatics and lymph nodes and eventually an invasion of the blood stream accompanied by severe toxic symptoms Endocarditis either acute or subacute, is a serious complication (Petersen et al 1950) Hamburger and Knowles 1953) The duration of the disease may vary from a period of a few days to one of several weeks In untreated cases the mortality rate is about 10 per cent

In the case of a food borne infection the portal of entry of the organisms is thought to be the intestines gastro intestinal symptoms are common However the rapid loss of viability of *Streptobacillus* cultures in a slightly acid environment casts serious doubt on this view The oral region or throat might also be considered as a possible portal of entry, *throat soreness is a frequent symptom*

In a comprehensive study (Freundt 1956a) mice were readily infected by the subcutaneous intraperitoneal intravenous and intranasal routes as well as by feeding and less readily by contact with rats The mice were infected but not easily through the conjunctival sac in this particular study In the feeding experiments it was definitely shown that invasion of the host by the micro organisms did not take place through the intestines but rather through the mouth or the throat Mice with different genetic backgrounds show different degrees of susceptibility (Mackie et al 1933)

In the acute stage the infection is apt to be of the septic type In the chronic type where the host may show greater resistance there is a greater tendency toward abscess formation and joint involvement *In experimental* infections in the developing chick embryo the organisms invaded the blood stream and localized almost exclusively in the synovial lining of the joints (Buddingh 1944) In the early stages of the development of the joint lesions *S moniliformis* was found to behave as a facultative intracellular parasite within the cytoplasm of the synovial lining cells While

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The Pleuropneumonia and Pleuropneumonialike Organisms

The micro-organisms which are designated as pleuropneumonia or pleuropneumonialike organisms are characterized by the lack of a rigid cell wall, a very pleomorphic cell morphology, and a characteristic colony morphology. Except for a few saprophytic strains, they require a culture medium enriched with fresh native body protein such as ascitic fluid or blood serum. In addition to growing on the surface of a solid medium, a portion of the colonies grows into it so that, unlike bacterial colonies, these cannot be readily rubbed off the surface of the medium. The cells are smaller than the usual bacterial cells and stain poorly with the ordinary aniline dyes. The colonies are best recognized by staining in situ with a mixture of methylene blue and azure II according to the method of Dienes. The organisms are inhibited by the action of specific antibodies in the absence of complement. Various generic names which have been proposed in previous systems of classification are Coccobacillus, Micromyces, Asteromyces, Borrelomyces, Bovimyces, and Asterococcus. The genus name now proposed is *Mycoplasma* and the type species is *Mycoplasma mycoides*.

For many years the pleuropneumonia group of micro-organisms was composed of the micro-organisms which cause pleuropneumonia of cattle and contagious agalactia of sheep and goats. Within the last two decades many micro-organisms which resemble the organisms of pleuropneumonia in colony morphology and cultural requirements have been isolated under various conditions from man and other species. These more recently isolated organisms, for the sake of convenience, have been referred to as pleuropneumonialike organisms or, for greater convenience, simply by the abbreviation PPLO. Insufficient knowledge of the numerous strains of PPLO is available to permit a precise classification. The latest proposals for classification and nomenclature are those of Edward and Freundt (1956), and their recommended terminology is represented by the names which appear in italics in the text.

HISTORY

The causative organism of bovine pleuropneumonia was discovered in 1898 by Nocard and co-workers. North America, Western Europe, and India are the only areas in the world in which bovine pleuropneumonia does not seem to exist. The disease was eradicated from the United States in the middle of the 19th century, and introduction of the micro-organisms into this country is prohibited by Federal law.

Since the small viable elements are capable of passing through bacteria-proof filters, the organisms were regarded by earlier workers as filterable viruses. In cattle the disease involves primarily the respiratory tract—pneumonia with secondary involvement of the pleura—with an occasional involvement of

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The Pleuropneumonia and Pleuropneumonialike Organisms

The micro-organisms which are designated as pleuropneumonia or pleuropneumonialike organisms are characterized by the lack of a rigid cell wall, a very pleomorphic cell morphology, and a characteristic colony morphology. Except for a few saprophytic strains they require a culture medium enriched with fresh native body protein such as ascitic fluid or blood serum. In addition to growing on the surface of a solid medium, a portion of the colonies grows into it so that, unlike bacterial colonies, these cannot be readily rubbed off the surface of the medium. The cells are smaller than the usual bacterial cells and stain poorly with the ordinary aniline dyes. The colonies are best recognized by staining in situ with a mixture of methylene blue and azure II according to the method of Dienes. The organisms are inhibited by the action of specific antibodies in the absence of complement. Various generic names which have been proposed in previous systems of classification are Coccobacillus, Micromyces, Asteromyces, Borrelomyces, Bovimyces, and Asterococcus. The genus name now proposed is *Mycoplasma* and the type species is *Mycoplasma mycoides*.

For many years the pleuropneumonia group of micro-organisms was composed of the micro-organisms which cause pleuropneumonia of cattle and contagious agalactia of sheep and goats. Within the last two decades many micro-organisms which resemble the organisms of pleuropneumonia in colony morphology and cultural requirements have been isolated under various conditions from man and other species. These more recently isolated organisms, for the sake of convenience, have been referred to as pleuropneumonialike organisms or, for greater convenience, simply by the abbreviation PPLO. Insufficient knowledge of the numerous strains of PPLO is available to permit a precise classification. The latest proposals for classification and nomenclature are those of Edward and Freundt (1956), and their recommended terminology is represented by the names which appear in italics in the text.

HISTORY

The causative organism of bovine pleuropneumonia was discovered in 1898 by Nocard and co-workers. North America, Western Europe, and India are the only areas in the world in which bovine pleuropneumonia does not seem to exist. The disease was eradicated from the United States in the middle of the 19th century, and introduction of the micro-organisms into this country is prohibited by Federal law.

Since the small viable elements are capable of passing through bacteria-proof filters, the organisms were regarded by earlier workers as filterable viruses. In cattle the disease involves primarily the respiratory tract—pneumonia with secondary involvement of the pleura—with an occasional involvement of

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the joints Joint involvement is a clinical sign which appears with varying degrees of frequency in a majority of the animal species infected with these organisms

In 1925 Bridre and Donatien isolated from sheep and goats the causative organism of contagious agalactia a disease found predominantly in Europe and Algeria The disease involves the joints and the eyes and in lactating sheep and goats the mammary glands Shoetensack in Japan in 1934 isolated similar organisms from dogs with a respiratory type of infection

Beginning with the middle 1930 s publications on these micro organisms began to appear more frequently in the scientific literature At first reports appeared on the isolation from man mice rats, fowl and guinea pigs Later the organisms were isolated from additional species of fowl and from horses swine and cats The rabbit appears to be the only common laboratory animal from which they have not been isolated

The discovery by Klieneberger in 1935 of the dissociation of *Streptobacillus moniliformis* into a small colony variant resembling the pleuropneumonia organisms and designated the L_1 variant initiated a great amount of investigation into the possibility of other bacteria producing similar variants and the possibility of finding the variants in various animals As the first variant was designated L_1 a similar terminology was employed for subsequent isolations This system of naming strains was discontinued after the L_7 term but the L designations still appear occasionally in the scientific literature The L terms had the following original connotations

L_1—the small colony variant produced by strains of *S moniliformis* and described by Klieneberger in 1935 Edward stated in 1950 that while the L_1 organisms have the general characteristics of PPLO they are quite distinctive in their cellular morphology and they frequently produce growth which resembles that of ordinary bacteria

L_2—the PPLO discovered by Klieneberger in 1935 to be associated with a small streptococcus or streptobacillus isolated from the nasopharynx of guinea pigs

L_3—the PPLO isolated by Klieneberger and Steabben in 1937 from the lungs of rats exhibiting chronic bronchopneumonia or bronchiectasis A similar strain was isolated from the brain of an apparently healthy mouse by Findlay et al in 1939 The name *Mycoplasma pulmonis* has been proposed for this strain

L_4—a term employed for the only strains of PPLO believed to possess pyogenic properties These organisms were isolated from rats by Klieneberger in 1939 and by Woglom and Warren in 1939 Arthritis is invariably produced when these organisms are injected into the foot pads of rats and mice The organisms are similar serologically to the L_7 organisms but they are more pathogenic for mice by intracerebral inoculation than the L_7 The name *Mycoplasma arthritidis* has been proposed

L_5—a term employed for the only strains of PPLO which produce rolling disease in mice The organisms were encountered by Findlay et al in 1938 during the brain to brain passage in mice of neurotropic yellow fever virus and the virus of lymphocytic choriomeningitis The intracerebral injection of pure cultures of the organisms did not produce rolling disease but the culture mixed with brain suspension containing any one of several viruses did produce the characteristic symptoms Mice and the field vole *Microtus agrestis* are the only susceptible animals The name *Mycoplasma neurolyticum* has been proposed

L_6—strains from PPLO isolated by Findlay et al in 1939 from the brains of mice The organisms are related to L_5 organisms but they differ from these in growth and serologic characteristics In morphologic and staining properties the organisms can be mistaken for *Eperythrozoon coccoides*

L_7—strains of PPLO isolated from arthritic joints of rats by Findlay et al in 1939 It was shown in 1940 by Klieneberger that the organisms are identical with the L_4 organisms

The first known encounter with PPLO in man is perhaps the work of Gey (personal communication) who succeeded about 1935 in isolating several dozen lines from human placental cord blood which was being employed in tissue culture work However the identity of these agents as PPLO was not possible until the special technics for working with these organisms were developed by Dienes Currently the PPLO are a problem in tissue culture work as many lines of cell cultures are contaminated with these organisms (Robinson et al 1956 Collier 1957 and unpublished work) Collier found the contaminating PPLO strains in the English

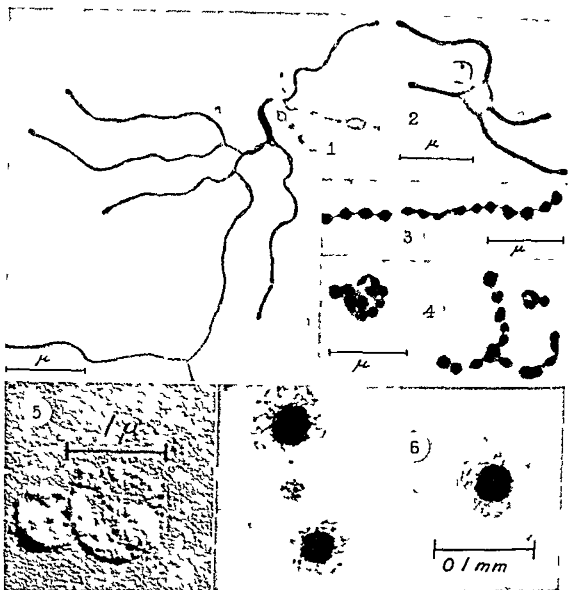


FIG 60 (1) Electronmicrograph of *Mycoplasma mycoides* from a 24 hour serum broth culture which shows homogeneous branching filaments with small terminal buds

FIG 61 (2) Electronmicrograph of *M mycoides* from a 24 hour serum broth culture which shows an elementary corpuscle and a moderately swollen germinating elementary corpuscle

FIG 62 (3) Electronmicrograph of *M mycoides* from a 24 hour serum broth culture which shows a filament with endomycelial elementary corpuscles

FIG 63 (4) Electronmicrograph of *M mycoides* from a 24 hour serum broth culture which shows an asteroid element and a short branching filament with endomycelial elementary bodies Figures 60 to 63 were published as Figures 4 6 9 and 10 by E A Freundt Acta Path et Micro biol *31* 508 529 1952

FIG 64 (5) Electronmicrograph of chromium shadowed cells of Campo strain pleuropneumonialike organisms from man from a 48 hour culture on solid medium This was reproduced as Figure 13 Morton et al 1954 J Bact *68* 697 717

FIG 65 (6) Photomicrographs of colonies of pleuropneumonialike organisms stained by the method of Dienes which were reproduced as Figure 1 by Leberman et al 1950 J Urol *6* 167 173 The colonies on the left are from a strain which was isolated from the human cervix The colony on the right is from a strain which was isolated from the male urethra as a pure culture

Sullivan and Dienes in 1939 encountered one strain of PPLO which grew only in embryos that had been killed by chilling and Swift in 1941 observed that in general PPLO grew better on dead sterile chorio allantoic mem branes than on living membranes Many of the strains from other species of animals are pathogenic for the embryos The organisms grow readily in tissue cultures and currently are an important factor as contaminants in tissue cultures employed for propagating viruses

Because of the frequent occurrence of PPLO along with bacteria in clinical materials the frequency with which cultures of PPLO be come contaminated with bacteria the small colonies produced by PPLO their slow rate of growth and their requirement for a rich medium it is often necessary to suppress the growth of bacteria while permitting PPLO to grow The organisms differ from either gram positive or gram ne ative bacteria toward selective bacteriostatic substances A combina tion of crystal violet and potassium tellurite may be employed to separate PPLO from bacteria Thallium acetate was al o employed by Edward in 1947 and appears to possess many advantages over other selective agents (Morton and Lecce 1953) Penicillin may also be employed if the bacteria to be in hibited are penicillin sensitive

Resting cells of PPLO are not very re istant to physical factors as compared with vegeta tive cells in general A typical strain of PPLO had a half life of 15 min 45 min 35 min 20 min and over 24 hours at 37 C when suspended respectively in distilled water 0 85 per cent NaCl solution M/15 phosphate buffer pH 7 0 Ringer s solution and the cul ture supernate Survival was found to be only slightly better at room temperature At 4 C the half life periods were more than 6 days in each case (Smith and Sasaki 1958) At 50 C the half life was 2 min or less no organ isms survived 7 to 10 minutes exposure at this temperature PPLO are much more sus ceptible than most bacteria to the deleterious action of agents which alter surface tension as evidenced by their susceptibility to the action of soaps and to the action of bile acids In 5 bile acids tested—cholic desoxycholic and lithocholic—a direct relationship was noted between the destruction of PPLO and the degree of surface tension depression due to the number of polar groups (Smith and Sa saki) Surprisingly PPLO were found to be relatively insensitive to osmotic shock This may be because of their pliable limiting cell membrane in place of the rigid cell wall found in bacteria In regard to their resistance to phenol hydrogen peroxide and ultraviolet light members of this group resemble bac terial vegetative cells (Warren)

Metabolically PPLO are a heterogenous group Many strains are inert toward carbo hydrates as shown by the usual fermentation tests Other strains from man and other species are capable of utilizing the test carbohydrates and do so with the accompanying production of acid and no gas (Edward 1954 Freundt 1954) Of 207 human strains of PPLO 189 were found to be biochemically inert in fer mentation tests 2 strains had fermentation reactions identical with the organism of bovine pleuropneumonia and the balance of the strains had variable fermentation reactions (Freundt 1954) The organisms have a very active monohydric alcohol dehydrogenase less dehydrogenase activity with lactate fructose and ribose and no activity with glucose De hydrogenase activity is also evident from the ability of the organisms to reduce methylene blue to a varying degree and to reduce tetra zolium compounds (Somerson and Morton 1953) In a synthetic medium the organisms appeared to derive their entire energy sources from amino acids and Sn^{++} served an essen tial role in some manner Attempts to demon strate transaminases have not been successful Utilization of arginine glutamine glutamic acid aspartic acid tryptophane and tyrosine and under aerobic conditions only histidine leucine and threonine has been demonstrated Among the limited biochemical activities of these organisms deamidation appears to be a significant function That of glutamine ap pears to be brought about by two distinct reactions One reaction is a simple hydrolytic deamidation and the other is a reaction which requires Mg^{++} phosphate and adenosinedi phosphate Glutamic acid is converted to a cyclic compound which is then reduced to pro line through the mediation of reduced triphos phopyridine nucleotide (Smith 1957a) Argi nine is converted to citrulline and this is converted to ornithine which is also utilized

tissue cultures to be antigenically similar by the complement fixation reaction to strains of PPLO from human genital infections

CULTIVATION AND BIOLOGIC PROPERTIES

PPLO are the smallest micro organisms which have been cultivated *on cell free media* Their nutritional requirements, except for the saprophytic strains, are different from those of all other micro organisms It is essential to start with a rich basal medium and to supply the additional growth factors

In most cases an infusion from beef heart *muscle is employed* as *infusions* from other tissues are less suitable To the infusion are added 0 5 per cent NaCl and appropriate peptone to the concentration of 1 per cent *The brand of peptone* is important, as not all brands support growth of PPLO equally well Bacto peptone was found to be the best of the available American peptones by Morton *Smith* and Leberman in *1951* For solid medium 1 4 per cent agar may be added The organisms are so sensitive that they may be inhibited by certain lots of bacteriologic agar (Lynn and Morton *1956*) *It* is desirable to have the medium as soft as practicable since the colonies grow into the medium

Since the organisms require an alkaline environment *for* optimum *growth* the final reaction of the medium should be about pH 7 6 to 7 8 The basal medium requires enrichment with a native body protein such as human ascitic *fluid* or *blood* erum *Ascitic* fluid in the final concentration of 20 to 30 per cent appears to be the best enrichment but often this is impractical Blood serum in *a final concentration of 10 to 20 per cent is* frequently used rabbit horse or beef serum are usually used but the serum from no one species is uniformly satisfactory

Often the sera of some species or certain lots of serum from the same species either do not promote growth or inhibit the growth of some strains This lack of a growth promoting *property in certain* sera may be due to the presence of specific antibodies Although beef serum is unsatisfactory as an enrichment for growing many human strains of PPLO a way was *found in* 1951 for separating the growth factor or factors from the toxic fraction (Smith and Morton) The complete basal medium in the dehydrated form and a serum fraction *containing* the growth factor in a concentrated form are available commercially so that cultivation of *PPLO* has become as practicable as cultivating many species of bacteria

The growth factor has been shown subsequently to be a lipoprotein containing only bound cholesterol and phospholipid This lipoprotein growth factor can be replaced with the lipid free protein which does not appear to be specific and cholesteryl laurate and lecithin of animal origin The lipid requirement for growth was also demonstrated by Edward and Fitzgerald in 1951 Only the amino acids alanine arginine, glutamic acid glycine, leucine lysine tryptophane and valine were found by Smith and Morton in 1952 in the protein component of the purified growth factor The growth factor is nonantigenic in rabbits (Smith, Morton, and Keller 1953) and is not known to be required by any other micro organisms Desoxyribonucleic acid was found by Edward and Fitzgerald in 1952 to be a growth factor for certain strains of PPLO when first isolated from cattle The addition of yeast extract to the culture medium may also enhance the growth of some strains

The growth of PPLO can be measured quantitatively by some of the methods commonly employed for estimating numbers of viable bacteria e g turbidimetric measurement of concentrated cell suspensions cellular nitrogen and counts of surface colonies re*sulting from the deposition of* measured amounts of cell suspension on the surface of appropriate solid medium (Smith 1956) Usually no noticeable turbidity is produced by the growth of *PPLO* in *liquid media The* amount of cellular nitrogen in broth cultures of PPLO is about 0 01 that of bacterial cultures

In general the organisms can be cultivated under aerobic conditions An occasional strain is encountered which grows only under an aerobic conditions but this may result from some inadequacy of the culture medium The presence of 10 per cent CO in the environment offers no advantages if the culture *medium is satisfactory and it may be* detrimental if the medium is slightly unsatisfactory Growth of the parasitic strains takes place at 37 C The organisms appear to re*produce by binary fission with a generation* time calculated to be 3 27 ± 0 78 hours Most of the strains of human origin grow in the developing chick embryo without producing noticeable pathology or death of *the embryo*

Regularly spaced constrictions take place in the walls of the filaments between the small bodies until finally the small bodies (elementary corpuscles) are held together by a delicate filament which is devoid of protoplasm. The elementary corpuscles are released when the constrictions completely sever the cell wall (Figs 60-63)

In a study of 207 human strains of PPLO Freundt (1954) found two which were identical with the organisms of bovine pleuropneumonia in their biochemical reactions and in their cellular morphology as revealed by the electron microscope. The majority of the strains (189) were characterized by a difference in morphology and a lack of biochemical activity. The initial mycelial forms of the organisms in this large group were rather stout, short and stiff. The fine delicate filaments so characteristic of the pleuropneumonia organisms were observed only rarely. With these organisms the filaments tended to fragment into cocci and irregularly shaped rods at an early stage of growth. The balance of the strains, about 7 per cent, were intermediate between these two groups in their morphology. Somewhat similar morphologic forms appear in electron micrographs of organisms causing agalactia and pleuropneumonia in goats (Klieneberger-Nobel and Cuckow 1955). Electron micrographs of human strains and of a poultry strain showed spherical bodies of less than 1 μ, some of which were undergoing division (Morton et al 1954) (Fig 64). Flagella never have been observed on cells of PPLO.

RELATIONSHIP OF PLEUROPNEUMONIA AND PLEUROPNEUMONIALIKE ORGANISMS TO BACTERIA

The pleuropneumonia and pleuropneumonialike organisms appear to be between the viruses and the rickettsiae on the one hand and the bacteria on the other. The pleuropneumonia organisms form a rather homogeneous group and they are distinct in their general properties from the viruses, the rickettsiae or the bacteria. The pleuropneumonialike organisms form a heterogenous group. Some strains resemble the pleuropneumonia organisms in their morphology and in their serologic and biochemical reactions while other strains have more of the properties of bacteria in their nutritional requirements and cellular morphology.

Klieneberger's discovery in the mid 1930's of the small colony variant (the L_1 variant) of *S moniliformis* which resembles the colonies of the pleuropneumonia organisms stimulated efforts to determine whether this new type of bacteria existed free in nature and whether similar forms could be produced from other bacteria. Impetus was also added to the work by the discovery that the L_1 variant is extremely resistant to penicillin. Not only can penicillin be used to separate the penicillin resistant L forms from the more penicillin sensitive bacterial forms when both are present in mixed cultures, but penicillin stimulates the production of L forms from bacteria. In their review Dienes and Weinberger (1951) listed 13 genera of bacteria from which L forms had been produced: *Bacillus, Bacteroides, Clostridium, Escherichia, Flavobacterium, Hemophilus, Neisseria, Pasteurella, Proteus, Salmonella, Shigella, Streptobacillus* and *Vibrio*. To this list can now be added *Azotobacter* (Eisenstark et al 1950), *Brucella* (Nelson and Pickett 1951), *Corynebacterium* and *Staphylococcus* (Dienes and Sharp 1956) and *Streptococcus*.

For practical purposes the micro organisms resembling the pleuropneumonia organisms which are isolated from various sources without any apparent relationship to a known bacterium are designated PPLO. When similar forms are isolated from a bacterial culture and bear some relationship to the culture, the forms are referred to as "L forms" and their colonies as "L type colonies." The demonstration of the characteristic colonies becomes the important criterion in determining whether one is dealing with L forms rather than with the pleomorphic large swollen forms of bacteria. The development of L type colonies is interpreted by Dienes and Sharp (1956) as evidence that viable granules in "L forms" have germinated. A definite relationship has been demonstrated for the L_1 variant and its parent culture of *S moniliformis* and between certain L forms and their parent *Proteus* cultures (Dienes, Weinberger and Madoff). In the case of one strain of *Streptococcus pyogenes* the L form continued to produce the type specific protein (type 19). In two strains

(Smith 1957b) The breakdown of glutamine and citrulline gives rise to the formation of adenosinetriphosphate

Colony morphology differs considerably from that of bacteria and varies less among strains within the group It is one of the properties which is detected most easily and is looked for early when attempting to detect these organisms The diameters of the colonies vary from approximately 10 μ to 1 mm but usually are about 250 to 750 μ Well isolated colonies are usually circular with an entire edge although Shepard (1956) reported tiny colonies (T forms) 10 to 12 μ in diameter which were irregular in outline Because of their small size the colonies are examined best with the low power of the microscope (100×) and transmitted light The colonies usually grow into the medium so they are not rubbed off when an inoculating loop is drawn over the surface of the culture Well isolated colonies developing under optimal conditions usually show the "fried egg appearance when viewed by transmitted light This appearance is caused by the fact that the central portion of the colony grows into the medium and the surrounding portion grows on the surface The appearance is shown in photographs published by Edward in 1950 and by Somerson and Morton

The surface of the colonies is raised and varies in texture from a slight pitting to a coarse lacy appearance as shown in photographs published in 1950 by Nelson and by Edward In addition there is sometimes a centrally located small peak (Sabin 1941) or depression (Edward) The colonies may be further identified by staining in situ by the method of Dienes (Fig 65)

Individual organisms are not stained readily by the dyes usually employed to stain bacteria and are gram negative Giemsa's or Wayson s stain is usually employed

Serologically the numerous organisms in this large group comprise many antigenic types Klieneberger in 1940 found distinct serologic types by the agglutination reaction The complement fixation reaction is proving to be the more practical serologic method for working with the human strains of PPLO as the organisms frequently produce suspensions which are unsuitable for agglutination tests Significant titers have been reported for sera from patients with deeply localized infections with PPLO (Melén and Gotthardson, 1955, Stokes, 1955) As in the case of other infectious diseases, animals that have recovered from infections may be immune to subsequent infections frequently without exhibiting specific antibodies in their sera (Edward 1954)

An interesting property of organisms of this group is that their growth is inhibited by specific antibodies in the absence of complement This characteristic is analogous to the neutralization test with viruses The growth of the L type variant of *Proteus vulgaris* was inhibited by its specific antibody but not by antibodies to the bacillary forms of *P vulgaris* This indicates important differences between the L and the bacillary forms of the same organism

The hemagglutination and hemagglutination inhibition tests are used extensively with the avian strains of PPLO

Interesting from the standpoint of the ecology of PPLO associated with man is the finding by Freundt (1954) that two strains are identical serologically with the organism of bovine pleuropneumonia and the finding by Smith Peoples and Morton (1957) that a poultry strain and a human strain cross react serologically

The knowledge of the morphology of PPLO is not as precise as that pertaining to bacteria partly because of the small size of the cells the poor staining with ordinary dyes and the fact that the colonies of PPLO are quite distinct Phase-contrast microscopy and electron microscopy have been helpful Freundt gave a good description in 1952 of the growth of the bovine pleuropneumonia organisms Both granules and filaments were demonstrated The smallest elements which are capable of germination and are filterable are the so called elementary corpuscles The formation of branching mycelia is also part of the morphology of these organisms and is a characteristic which differentiates them from bacteria Growth has been observed to take place by the extrusion of thin filaments from the elementary corpuscles These filaments are without septa and may reach a length of 150 μ As the organisms become older these homogenous filaments become transformed into long chains of regularly spaced closely set highly refractive uniformly shaped small bodies

Regularly spaced constrictions take place in the walls of the filaments between the small bodies until finally the small bodies (elementary corpuscles) are held together by a delicate filament which is devoid of protoplasm. The elementary corpuscles are released when the constrictions completely sever the cell wall (Figs 60-63).

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studied the L forms failed to produce the group specific polysaccharide (Sharp et al, 1957)

The L forms isolated from various strains of bacteria differ considerably, and the L forms isolated from the same strain of bacterium may differ L forms frequently are more difficult to cultivate in liquid media than are PPLO (Dienes 1953), and this property is sometimes employed as a means of differentiating the two types of organisms However Medill and O Kane (1954) grew the L forms of *Proteus* in a synthetic liquid medium and suggested that a reason for poor growth in natural media might be the presence of an inhibitor or inhibitors in some of the natural products Serum or plasma might function as a detoxifying agent rather than as a supplier of necessary nutrients Nevertheless Edward pointed out in 1953 that there may be differences in the nutritional requirements of L forms and PPLO

The L forms may arise spontaneously in bacterial cultures or they may be produced when actively metabolizing bacterial cells are subjected to some unfavorable condition The substances which have proved to be useful in producing L forms are penicillin and a few other antibiotics glycine and a few other amino acids (Dienes and Zamecnik 1952) antibodies in the presence of complement bacteriophage and such unfavorable conditions for normal bacterial growth as are brought about by high concentrations of electrolytes (Dienes and Sharp 1956) refrigeration bacterial antagonism and tap water

A better understanding of the relationship of certain micro organisms to disease may come about if it is established that some of the PPLO isolated from the body are related to bacteria especially to pathogenic bacteria Since the L_1 variant of *S moniliformis* has been isolated from rats in the absence of *S moniliformis*, there may be comparable circumstances in the case of other bacteria and man The Campo strain of PPLO which was isolated from the human urethra has been maintained on artificial medium for over 10 years During the past years liquid cultures of this strain as well as those of another strain from the human urethra have been observed repeatedly to contain *Corynebacteria* (Smith et al 1957) Statistically it appears very unlikely that the *Corynebacteria* are contaminants and moreover the PPLO and the associated *Corynebacteria* are related serologically and have similar fermentation reactions Wittler et al (1956) obtained *Corynebacteria* from a urethral strain of PPLO by cultivating the latter in tissue cultures These strains of *Corynebacteria* were identical serologically and biochemically with a strain of *Corynebacterium* originally isolated from the same urethral exudate at the time the PPLO were isolated and were related antigenically to that strain of PPLO The biochemical properties of these strains of *Corynebacteria* are different from those of the *Corynebacteria* isolated from cultures of PPLO by Peoples et al Wittler et al suggested that with further study other human genital strains of PPLO may be found to be L forms of *Corynebacterium* spp Amies and Jones (1957) present a similar view in regard to the relationship of certain PPLO from the genito urinary tract to *Haemophilus vaginalis* In the field of veterinary medicine a comparable view had been expressed by McKay and Taylor (1954) when these workers recovered from PPLO associated with chronic respiratory disease of chickens and sinusitis of turkeys a gram negative rod which resembled *Haemophilus gallinarum* They suggested that the agent of these infections was the L form of a true bacterium and not a PPLO

DISTRIBUTION IN NATURE EPIDEMIOLOGY AND RANCE OF PATHOGENICITY

It is now apparent from studies during the past few years that PPLO are not only very prevalent in man during health and disease but are prevalent in many species of animals with which man has contact The finding that some of the strains of PPLO isolated from man react serologically with strains isolated from other species of animals indicates that more needs to be known about the ecology of these micro organisms in order to understand better their role in disease

Man The first isolation of PPLO from a pathologic process in man was from the genito urinary tract by Dienes and Edsall in 1937, and the greatest amount of work during the past 20 years in relation to the human strains

of PPLO has pertained to the study of their prevalence, and nature and their possible relationship to pathology in that region The organisms are more apt to be present in the genito-urinary tract if some pathologic condition is present This association was found by Melen and Odeblad in 1951 to be statistically significant In culturing the urethra the vagina and the cervix of 58 gynecologically normal women ranging in age from 16 to 44 years these investigators in 1952 failed to find the organisms in 13 virgins (mean age 21 years) although Freundt found the organisms in cultures from the vulva of 3 of 50 girls under 3 years of age and in the vaginal cultures from 4 of 6 adult virgins If a single area or combination of any of the three areas—the urethra the cervix or the vagina—is positive for PPLO it invariably is the vaginal culture in which the PPLO appear Therefore cultures from the vagina will give a good indication if PPLO are present in the lower female genital tract The fact that PPLO grow better in an alkaline environment may be an explanation for their scarcity in the healthy vagina Freundt found a distinct relationship between the pH of the vagina the vaginal flora and the presence of PPLO The PPLO were usually absent in the vaginas with the lower range in pH (3 7 to 5 0) and the flora exclusively Doderlein s bacillus A similar relationship between the presence of PPLO and lactobacilli in the vagina was noted by Huijsmans Evers and Ruys (1956) Morton et al (1952) failed to culture the organisms from the cervix of 12 healthy married women while Melen and Odeblad in the same year found the organisms in 5 of 32 nonpregnant married women and in 3 of 13 pregnant women Klieneberger Nobel in 1945 cultured the organisms only twice and then there was only sparse growth from 45 normal pregnant women in contrast with finding the organisms in 40 per cent of 45 women attending a venereal disease clinic

The association of PPLO and *Neisseria gonorrhoeae* in cervical cultures was found by Somerson et al (1955) to be statistically significant and no such relationship was found between the presence of PPLO and leukorrhea gross genital pathology phase of menstrual cycle or previous penicillin therapy in the group of 86 women studied The apparent association of the presence of PPLO and previous penicillin therapy is of interest because of the ability of penicillin to produce the L forms of many bacteria and to reduce the number of penicillin sensitive organisms in the normal flora Schaub and Guilbeau in 1949 isolated PPLO from the postpartum uterus of 3 per cent of the untreated patients and from 20 per cent of the patients who had received penicillin In women who had received penicillin 70 per cent of the cervical cultures yielded PPLO while 26 per cent of 300 consecutive gynecologic patients and 17 per cent of the women with noninflammatory diseases were positive for PPLO (Randall Stein and Ayres)

Various other pathologic conditions in the female genito urinary tract from which PPLO have been isolated are chronic vulvitis (Homma and Kusano 1954) fusospirillary vulvovaginitis vaginitis cervicitis and cystitis (Dienes Ropes Smith Madoff and Bauer) and inflamed uterine tube and salpinx

The finding in 1946 by Salaman et al of PPLO in urethral discharges from cases of nongonococcal urethritis stimulated investigations into the possible etiologic role of these organisms in this condition the incidence of which now exceeds that of gonorrhea The etiology of nongonococcal urethritis remains obscure but it appears that more than one agent may be responsible for it The situation is further complicated by the fact that frequently PPLO are isolated from the normal apparently healthy male urethra Morton et al (1952) failed to isolate PPLO from the semen of 28 healthy individuals who were being studied in a fertility research laboratory but Freundt (1956a) in Denmark isolated PPLO from the urethra of 15 ([illegible] of 28 healthy (trivial skin complaints [illegible] healthy as regards their genitals) [illegible] compared with an incidence of [illegible] per cent from cases of nongonococcal [illegible] per cent from cases of abacterial [illegible] cal urethritis 34 per cent from cases [illegible] gonorrhea and 47.8 per cent [illegible] epididymitis without urethritis The [illegible] Shepard introduce [illegible] complex situation [illegible] urethral cultures o [illegible] per cent [illegible] Negro male medical [illegible] cent of 55 normal [illegible] and staff [illegible] student who [illegible] one of 24 [illegible] Of the 3[illegible] Negro [illegible] urethr[illegible] PPLO [illegible] cent and [illegible] 56 per cent [illegible] [illegible]

respectively Against these data one has to weigh the evidence that in many instances PPLO are the only organisms or the predominating organism cultivated from a pathologic process and when the organisms disappear during the course of therapy there is also a remission of symptoms Three patients with urethritis were treated successfully with streptomycin and 7 patients were treated successfully with oxytetracycline 2 of 3 patients responded to streptomycin therapy and 1 patient with urethritis of long duration was treated successfully with streptomycin (Melen and Linnros) Recovery following treatment with chlortetracycline was more frequent in those patients in whom PPLO could be demonstrated than in those in whom PPLO could *not be demonstrated (Jensen 1954)*

Other pathologic conditions of the male genito urinary tract from which PPLO have been isolated are fusospirochetal gangrene of the penis by Ruiter and Wentholt in 1950 phagedenic ulcer on young man with paraphimosis ulcerative balanitis with inflammatory phimosis and erosive balanitis by Ruiter and Wentholt in 1952 cystitis prostatitis and epididymitis

The strains of PPLO isolated from the *genito urinary tract of man are diverse in their* antigenic structure To one group of strains Edward and Freundt (1956) have given the name *Mycoplasma hominis* types 1 and 2 The majority of the strains which were isolated in England as well as some strains which had been isolated in France and the United States could be classified provisionally as type 1 Several strains all of which had been isolated in the United States differed from the type 1 strains and were classified *provisionally as type 2 Five strains studied by* Norman et al were serologically similar The strains of PPLO isolated by Ruiter and Wentholt from cases of balanitis and fusospirillary vulvovaginitis which differed culturally and serologically from the usual human genital strains and were designated 'G strains were classified as human type 3 strains and called *Mycoplasma fermentans* Genito urinary strains are encountered which cannot be classified into the above types (Huijsmans Evers and Ruys 1956)

Venereal transmission must be considered as a possibility when the organisms are present in either the male or the female genito urinary tract Dienes and Smith in 1942 reported two instances where there was the possibility of transmission of PPLO between husband and wife during intercourse in a third case a young girl developed an acute vaginitis 4 days after intercourse and an abundant growth of PPLO was obtained from the discharge Two additional instances were reported by Morton et al in 1951 in which similar PPLO were isolated from both the husband and the wife *The same type of PPLO were isolated by* Ruiter and Wentholt from a woman with severe leukorrhea and from the prostatic fluid from her husband and Melen and Linnros obtained a profuse growth of PPLO from the urethral vaginal and cervical cultures from the wife of a man with nongonococcal urethritis Schaffarzick and Mankle (1953) have reported a condition where it appeared that the male harbored an infection with PPLO transmitted the infection to a female contact *and later was reinfected by her With venereal* diseases in general both sexual partners must be taken into consideration and it now appears that in the case of suspected infections of the genito urinary tract due to PPLO both partners must be examined If PPLO are present in both both should be treated

PPLO were cultivated from the oral region in 1951 when Morton et al detected the organisms in the saliva of 46 of 100 individuals and Smith and Morton detected them in the *throat cultures of 38 of 114 individuals* Freundt isolated them from throat washings of 43 per cent of 95 women Although the association of the presence of PPLO in the oral region and the presence of the organisms in an infectious process may be coincidental two reports are of interest Paine et al in 1950 isolated an alpha streptococcus and a PPLO from a brain abscess in an individual who had the stem of a smoking pipe thrust into the brain during an altercation and Carlson *et al in 1951 cultured* PPLO from the blood of a child who gave a history of a human bite PPLO were isolated in pure culture postoperatively from a bronchopleural fistula after the removal of a drainage tube and later from fluid in the pleural cavity from a patient with bronchiectasis (Stokes 1955) The oral strains of PPLO differ from strains isolated from the genital tract in agglutination reactions colony morphology and nutritional requirements (Dienes and Madoff Nicol and Edward) The name *Mycoplasma salivarium* has been proposed for the strains of PPLO which have been isolated from the oral region of man

Likewise PPLO have been cultivated from the lower end of the alimentary tract Harkness isolated PPLO 8 times from 52 individuals by means of anal swabs and swabs taken at sigmoidoscopy Morton et al (1952) iso

lated PPLO 3 times from feces from 27 individuals and Freundt isolated the organism from the rectal swabs of 7 of 25 women

No particular strain of PPLO from man has yet been demonstrated to have pyogenic properties as in the case of certain rat strains but PPLO have been associated with certain suppurative processes in man PPLO have been cultivated from abscessed Bartholin s glands in 6 cases a pelvic abscess associated with puerperal infection and a pelvic abscess associated with salpingitis (Dienes Ropes et al) a tubo ovarian abscess (Randall Stein and Ayres) and ovarian abscess in 2 patients and a periurethral abscess in a female patient (Melén 1952) in addition to 2 cases of brain abscess

Only 1 case of isolation of PPLO from an infection of the skin has been reported This infection a mixed infection with PPLO spirillae and fusiform bacilli was in and around the umbilicus of an obese woman in whom PPLO were also present in a leukorrhoic exudate The skin and the genital strains of PPLO differed in several respects (Ruiter and Wentholt 1955)

Reports of involvement of the human central nervous system have not been numerous Mention has been made of the isolation of PPLO from brain abscesses in an individual injured with the stem of a smoking pipe and in a child PPLO were the only organisms cultured from spinal fluid from the case of meningitis that developed in an infant with a sacral meningocele which ruptured during birth (Davis and Arnstein 1953)

Cultivation of PPLO from the blood has been reported in a few cases and in these instances the bacteremia usually has been associated with a pathologic condition in some other part of the body Carlson et al in 1951 isolated the organisms from the blood cultures of 3 children Two of the children had purpura a splinter in the finger of one child developed into an indolent ulcer the second child experienced a human bite and had an intussusception of the ileum The third child had a brain abscess and meningitis Slingerland and Morgan (1952) isolated PPLO from the blood stream and cervical secretions during a febrile illness which had its onset 24 hours after child birth The appearance of the organisms in the blood stream at the onset of the acute illness their persistence in the blood for over 24 hours and their disappearance with recovery of the patient suggest the organisms were the cause of the acute illness Stokes (1955) also reported a similar case of puerperal sepsis

Cultures of fluids from inflamed joints of individuals with PPLO in other regions of the body have yielded PPLO only occasionally as compared with the findings in animals Dienes Ropes et al cultured PPLO from the synovial fluids of 2 patients with Reiter s syndrome and Brown et al (1951) cultured the organisms only once from synovial fluids Kuzell and Mankle (1950) reported isolating PPLO from the joint fluid in 5 cases of Reiter s syndrome and more recently the number has been extended to about 10 cases (personal communication) The organisms have also been isolated from knee joint fluid from 2 cases of polyarthritis in women (Butas 1957)

It is only natural that interest should be aroused in the possible presence of PPLO in the conjunctivae of cases of Reiter s syndrome the triad of symptoms of urethritis arthritis and conjunctivitis Frequently PPLO are isolated from the urethral discharges of patients with Reiter s syndrome and successful isolations of PPLO from fluids of arthritic joints are becoming more frequent PPLO were isolated by Kuzell and Mankle (1950) from the conjunctivae of 5 cases of Reiter s syndrome and now the series has been extended to about 10 cases (personal communication) Two of 3 patients with Reiter s syndrome gave significant agglutinin titers with an antigen prepared from one strain of PPLO (Wallerstein et al 1946) With the currently improved methods of cultivation and of performing serologic tests the etiologic role of PPLO in Reiter s syndrome may now be investigated with better chances of success

The experimental determination of pathogenicity of the strains of PPLO from man is fraught with difficulties It is the usual finding that PPLO exhibit pathogenicity by experimental inoculation into only the species of animal from which the organisms were isolated or at the most there is a limited range of susceptible hosts Also many of the common laboratory animals are carriers of their own types of PPLO However there are a few instances in which certain strains of PPLO from man have shown some pathogenicity for experimental animal Two strains of PPLO were isolated by Ruiter and Wenthold in 1952 from cases of balanitis These differed from strains of PPLO isolated from cases of urethritis in colony morphology and cultural properties and were designated as G strains Inoculation of these organisms into the plantae of the hind legs of young white mice produced

pathologic changes in the tibiotarsal joints from which the organisms could be cultivated. In one case the knee joint and its surrounding musculature were involved. Virulence of the organism was enhanced by serial passage through the mice. No reaction could be elicited in the 2 patients when broth cultures of the 2 strains were injected intradermally. Likewise, no reaction occurred when a washed suspension of the organisms was introduced into the preputial pouch of 2 healthy men. In a case of phagedenic ulcer associated with paraphimosis, the intradermal injection of the necrotic material into the skin over the abdomen produced superficial ulcers. From these fusiform bacilli and spirilla but no PPLO could be demonstrated. Another G strain of PPLO was isolated from a case of fusospirillary vulvovaginitis and was similarly slightly pathogenic for young white mice. Two strains of PPLO isolated from men with urethritis failed to show pathogenicity when injected subcutaneously into the pads of the hind feet of young white mice. Additional evidence of the properties of communicability and pathogenicity of PPLO for man are unpublished observations of Kuzell that 2 laboratory workers became infected with the strains of PPLO which they had isolated from patients with Reiter's syndrome, and that a clinician had developed conjunctivitis and arthralgia following the examination of patients with a tentative diagnosis of Reiter's syndrome.

Cattle. The causative organism of bovine pleuropneumonia was cultivated by Nocard and co-workers in 1898 and studied by Bordet and by Borrel et al. in 1910. This organism is the type species, and the name *Mycoplasma mycoides* var. *mycoides* has been proposed. In 1950 Edward isolated 2 groups of pleuropneumonialike organisms from the genital tract of cattle. One group of strains, provisionally designated P strains, for which the name *Mycoplasma bovigenitalium* has been proposed, was thought to be capable of causing an inflammation of the bovine genital tract which predisposes to infertility. The strains were antigenically heterogenous, but they shared some antigens in common. They were antigenically distinct from the S strains and the organism of pleuropneumonia. Sera from infected animals did not show agglutinins for these strain. The other group of strains isolated from the genital tract of cattle, provisionally designated S strains, resembled the saprophytic PPLO in not requiring serum for growth and in growing at room temperature. They reacted serologically with the saprophytic strains from sewage, were antigenically heterogeneous, and were not related antigenically to the P strains or to the organism of pleuropneumonia. It was thought that these strains were commensals or contaminants in the discharges. PPLO and various species of bacteria have been isolated from the lungs of calves with the bronchopneumonia associated with the disease commonly referred to as shipping fever (Carter 1954), but the etiologic role of these organisms remains to be proved.

Man may have more intimate contact with strains of PPLO from bovine sources than is generally realized, as Alstrom (1955) found these organisms present in the milk from diseased and supposedly healthy cows in herds in which there was clinical mastitis. Furthermore these organisms were reported to be resistant to pasteurization.

Mice. A communicable disease among white mice, designated infectious catarrh, was described by Nelson in 1937 as being caused by "coccobacilliform bodies." The similarity between the coccobacilliform bodies and PPLO is so close that in 1950 Nelson stated there is little reason for separating them. The organisms involved in the catarrhal condition tended to multiply throughout the respiratory tract, particularly in the middle ears, and were referred to as the catarrhal strain. In the same year Nelson also described an outbreak of conjunctivitis unaccompanied by involvement of the respiratory tract in a colony of white mice. The organisms were referred to as the conjunctival strain as they exhibited marked differences from the catarrhal strain and also differed from the organisms described by Sabin. A high percentage of adult mice carried these organisms in their eyes, but only a very small percentage of the carriers showed an inflammatory reaction. Transmission to the young was thought to be by parental contact after the eyes became opened and continued after weaning by direct contact with cage mates. The organisms were also isolated from the conjunctivae of 8 per cent of wild house mice. Organisms of the catarrhal type selectively localized in the genital tract after intraperitoneal injection of female weanling mice, but organisms of the conjunctival type failed to survive in the abdominal cavity and produced no reaction in the mice (Nelson 1954). The growth of the catarrhal and conjunctival strains of PPLO was greatly enhanced in the brains of Swiss mice but not in Princeton mice by the simultaneous intracerebral injection of mouse hepatitis virus obtained from Balb C mice. The growth and the pathogenicity of the

catarrhal and the conjunctival strains of PPLO were greatly enhanced in the brains of Princeton mice but not in Swiss mice by the simultaneous intracerebral injection of mouse hepatitis virus obtained from Princeton mice (Nelson 1957)

During the brain to brain transfer of *Toxoplasma* in mice Sabin in 1938 in the United States encountered a new infectious agent with neurolytic properties The intracerebral injection of the agent into mice produced a characteristic turning of the mice on the long axis of the body Later in the same year Sabin demonstrated the agent to be PPLO which elaborated an exotoxin From the brain of one of 10 normal stock mice a strain of PPLO was isolated During the following year this strain was found to produce arthritis in practically 100 per cent of the mice into which it was injected intravenously or intraperitoneally Also in 1938 Findlay et al in England isolated from the brains of mice during the intracerebral passage of the virus of lymphocytic choriomeningitis a strain of PPLO designated L_5 which produced the characteristic rolling symptoms observed years earlier This strain and that of Sabin were identical serologically but differed in pathogenicity (Sabin 1941)

PPLO have been isolated from the brain the conjunctivae the nasal mucosa and the lungs of normal mice It is evident that mice frequently are carriers of PPLO and the organisms comprise a variety of immunologic and biologic types This can introduce complications in experimental work with mice such as the finding by Edward in 1947 of PPLO in the seed culture of *Rickettsia orientalis* which had been obtained by the intranasal passage of material in mice

Rats PPLO have been isolated from the lungs of rats exhibiting chronic bronchopneumonia or bronchiectasis (the L_3 organisms described by Klieneberger and Steabben in 1937 and 1940) but the investigators were unable to produce acute pulmonary disease with the cultures by injection The organisms were isolated in 1939 from rats exhibiting polyarthritis by Findlay et al and in 1940 by Nelson from rats showing an infectious catarrhal condition The pyogenic virus of the rat which was described by Woglom and Warren was identified as the L_4 strain of PPLO in 1939 by Klieneberger and Woglom and Warren The L_1 variant of *S moniliformis* has been found in rats independently of *S moniliformis*

Sheep The disease known as contagious agalactia was demonstrated by Bridré and Donatien in 1925 to be caused by microorganisms resembling those of pleuropneumonia of cattle Although agalactia is a systemic disease the joints the eyes and in lactating sheep and goats the mammary glands are affected The name *Mycoplasma agalactiae* has been proposed for the causative organism Recently strains of PPLO which are different from *M agalactiae* have been isolated from the lungs of sheep in North America (Greig 1955 Adler et al 1956) These strains were infectious and lethal for chick embryos but were not infectious for mice guinea pigs and rabbits Since the parenteral inoculations of the strains into a limited number of sheep were without noticeable effect the etiologic role of these strains for disease in sheep remains to be demonstrated The situation may be similar to that in which Beveridge in 1941 isolated PPLO from cases of foot rot in sheep but showed that the organisms had no causal relationship with the disease (Greig 1955)

Goats There are at least 2 and possibly 3 diseases of goats which are thought to be caused by PPLO Contagious agalactia was discovered by Bridre and Donatien in 1925 as being caused by pleuropneumonia organisms for which the name *Mycoplasma agalactiae* has been proposed (Edward and Freundt 1956) Later it was discovered (Longley in 1951 Durusan et al 1952) that a contagious pleuropneumonia of goats was not caused by a filterable virus as originally thought but rather by PPLO for which the name *Mycoplasma mycoides* var *capri* has been suggested The pleuropneumonias of goats and cattle are similar in their pathology and the organisms of the two diseases have similar cultural properties By means of the complement fixation test Edward in 1953 was able to demonstrate that the two species of organisms are serologically distinct but that the two may share common antigens More recently a highly fatal disease characterized by septicemia and arthritis was detected for the first time among dairy goats on the North American continent Goats sheep and a pig were susceptible but mice guinea pigs and a calf were resistant to experimental inoculation However the organisms were highly virulent to chick embryos (Yamamoto et al 1955)

Swine PPLO have been isolated from the turbinates of normal swine and swine suffering from infectious atrophic rhinitis (Switzer 1955) These organisms when inoculated intranasally into baby pigs frequently become established in the nasal cavity but do not

produce gross atrophy of the nasal turbinates Inoculated intraperitoneally into young pigs, the organisms produce fibrinous peritonitis pleuritis pericarditis and arthritis Carter in 1954 pointed out that these lesions resemble those of Glasser s disease and he too, was able to reproduce them by the intraperitoneal injection of cultures of PPLO Pigs over 6 weeks of age were observed by Switzer to show milder lesions From 5 to 20 per cent of the inoculated pigs developed arthritis The organisms can be grown in embryonated chicken eggs with about one half the embryos developing lesions or dying The swine PPLO were found to be avirulent for mice guinea pigs rabbits chickens turkeys a calf and a lamb The organisms do not produce acid from glucose maltose sucrose lactose or mannite they will withstand 56° C for 30 minutes but not for 1 hour Swine PPLO probably play a secondary role to a virus in producing pig pneumonia (Carter and Schroder 1956) The name *Mycoplasma hyorhinis* was proposed for these organisms, and their natural habitat appears to be the nasal cavities of swine

Dogs Shoetensack in Japan in 1934 isolated from dogs with a respiratory type infection organisms which were similar to the organisms that cause pleuropneumonia in cattle The name *Asterococcus canis* was suggested for these newly isolated organisms In 1951 Edward and Fitzgerald in England isolated PPLO from the vaginal smears of 54 per cent of the dogs examined at a kennel and from the semen of a dog being investigated for sterility and epididymitis There was evidence that the organisms could be transmitted venereally by the dogs These authors also isolated PPLO from the throat specimens of 74 per cent of the dogs examined On the basis of colony morphology practically all of the canine strains of PPLO could be classified into 3 types designated α β and γ This classification into 3 types was verified by serologic studies The α strains never were detected in throat specimens from the dogs but the 3 types were isolated from the vagina The 3 types were isolated once from one vaginal specimen Pathogenicity studies were not made The PPLO isolated by Greig (1951) from dogs in Canada with kennel cough produced little if any effect in dogs upon intranasal subcutaneous and intravenous inoculation The names *Mycoplasma spumans M canis* and *M maculosum* have been proposed for the α β and γ strains isolated from dogs An interesting observation is that of Dienes in 1939 in which he isolated a *Flavobacterium* and L type colonies from the dog bite wound of a patient How the organisms were introduced into the wound remains unknown

Avian Species One of the areas of very active research in the relation of PPLO to infectious diseases is the general field of poultry science A limited investigation demonstrated a serologic relationship between a strain of PPLO isolated from chickens and a strain isolated from man (Smith et al, 1957) which indicates the advisability of studying the ecology of PPLO Based on the study of only one species, the name *Mycoplasma gallinarum* has been proposed However, the relationship of this strain to those isolated from avian species, particularly chickens and turkeys in the United States and Canada remains to be studied

In extended studies of coryza in fowl started in the early 1930 s Nelson cultivated coccobacilliform bodies which were later considered to be PPLO The coryza of slow onset and long duration (coryza II) was shown to be caused by the coccobacilliform bodies whereas the coryza of rapid on et and short duration (coryza I) was caused by *H gallinarum* Coryza III had a rapid onset and a prolonged course and was found to be a mixed infection with *H gallinarum* and coccobacilliform bodies These interesting studies were confirmed by Adler and Yamamoto (1956b) Fahey and Crawley (1956) likewise presented evidence which they believed proved that infection of chicks with PPLO was mild in nature and was indistinguishable from coryza II The organisms of chronic respiratory disease of chickens and of infectious sinusitis of turkeys are pathogenic for the developing chick embryo (Markham and Wong)

It has been demonstrated that infected hens transmit their PPLO to the egg and thus infect the embryos The PPLO and the host have established a state of reasonably peaceful coexistence so that only a small percentage of the embryos and young chicks are killed The carrier chicks infect their penmates during the growing period by means of aerosols or contact The infection remains chronic in the adult birds and a small percentage of the adults pass the organisms through the egg to perpetuate the infection (Crawley and Fahey 1957)

If an infection with the virus of chronic respiratory disease or the virus of infectious bronchitis is superimposed upon the mild infection with PPLO a much more serious infection results To bring under control the severe chronic respiratory disease of chickens it is

necessary to break the cycle of transmission of PPLO from hen to egg This is done preferably by the parenteral administration of antibiotics (Adler et al 1956) Thus in order to control the severe chronic respiratory disease in chickens it is necessary to bring under control one of the organisms involved in the mixed infection and the control of PPLO afford the most practical solution

A hemagglutination inhibition (HI) test has been devised as a diagnostic test for infection with PPLO in chickens and in turkeys (Fahey 1954) The PPLO strains which are associated with chronic respiratory disease in chickens and infectious sinusitis in turkeys appear to be antigenically homogeneous with respect to this test and identical in morphology as shown by electron micrographs The continuous administration of either chlortetracycline or oxytetracycline in the feed does not free chickens from their PPLO and the antibiotics either retard or completely suppress the production of HI antibodies (Fahey and Crawley, 1955)

Pigeons with a mild respiratory disease have yielded PPLO in cultures of the nasal discharges (Mathey et al 1956) The organisms did not ferment any of several carbohydrates which were tested, and they differed in antigenicity and pathogenicity for chickens and turkeys from strains of PPLO which have been encountered in these 2 species

The isolation of PPLO from air sac exudate from a parakeet suffering from mild aerosacculitis introduces the possibility that isolating psittacosis virus from these birds may be more difficult (Adler 1957) The parakeet strain of PPLO was different antigenically from strains isolated from chickens and turkeys The organisms multiplied in the developing chick embryo but produced no mortality

PPLO and a virus were isolated by Fahey in Canada from ducks exhibiting a chronic respiratory disease The duck strain of PPLO fermented glucose and maltose weakly but did not ferment sucrose mannite and lactose The role of these organisms in producing disease in ducks remains to be elucidated

Guinea Pigs PPLO designated L_7 were isolated from the nasopharynx of healthy guinea pigs by Klieneberger in 1935 and from abscesses in guinea pigs (Klieneberger 1939 Findlay et al in 1940)

Horses There is one report of the presence of PPLO in the equine species Theiss (1947) isolated the organisms from an aborted fetus vaginal secretions and uterine discharges

Cats Recently PPLO have been isolated from the conjunctivae of domestic cats (Yerasimedes and Smith unpublished observations)

Saprophytic Strains PPLO were isolated by Laidlaw and Elford in 1936 from all samples of raw sewage from 4 districts of London during the summer but not from London tap water or from fecal material of man rabbit rat or pig Optimum growth took place at temperatures of about 30 C in media with a reaction of about pH 8 0 and although a very nutritious medium was required enrichment of the medium with a native body protein was not essential for growth Similar organisms were isolated from soil manure and compost by Seifert in 1937 in Germany Saprophytic strains have been isolated also from the bovine genital tract The name *Mycoplasma laidlawii* has been proposed for these strains

PATHOGENESIS

Little is known about the pathogenesis of infections with PPLO in man There is evidence that under certain conditions PPLO may initiate or contribute to a pathologic condition Furthermore when the organisms are eliminated from a particular area of the body by means of appropriate therapy the pathologic condition frequently disappears So little work has been done employing the direct inoculation of PPLO into humans that inferences on the behavior of human strains have to be drawn from work with other strains of PPLO in other hosts

The L_1 variant of *S moniliformis* is only slightly if at all virulent for mice whereas the bacillary form of *S moniliformis* is virulent (Freundt 1956b) This may indicate that in general PPLO may be expected to be less virulent than bacteria However some of the human strains of PPLO have been demonstrated to be slightly virulent for mice With organisms of slight virulence a lessened resistance on the part of the host will play an important part in determining whether or not an infection is established

When the organisms are introduced into the blood stream as a result of trauma such as that accompanying childbirth sepsis occasionally develops Since growth of the organisms is inhibited by their reaction with antibodies it can be expected that they will remain in the blood stream only until specific antibodies appear Contrariwise when the organisms are introduced into the central nervous system

where the immune reaction of the tissue is poor infection may result In general it is to be expected that the organisms will be able to establish themselves on surfaces of the body or in foci where the environment is suitable for their multiplication This is evident from the reports of their isolation from abscesses and inflamed mucous membranes

Some strains such as those from the mouse show a predilection for certain tissues, e g, the respiratory tract, the conjunctiva and the female genital tract It is not known whether any of the human strains show a similar specific tissue tropism

Mixed infections play an important role in enabling some strains of PPLO to proliferate in certain animal species and similar circumstances may be operating in man Mention has been made of the enhancement of the growth and the pathogenicity of PPLO in mice infected with mouse hepatitis virus Rolling disease a new disease of mice was produced by Findlay et al in 1938 at first only with a specific strain of PPLO (L_5), together with some other nonspecific agent The nonspecific agent could be either the virus of lymphocytic choriomeningitis or of yellow fever (neurotropic strain) and climatic bubo or a suspension of serum agar culture medium Later, by enhancing the virulence of the L_5 strain by rapid brain to brain transfer in mice rolling disease could be produced directly by injection of the culture PPLO have been shown also to proliferate more readily in mice infected with ectromelia virus (Schauwecker 1947) An infection with the virus of either chronic respiratory disease or infectious bronchitis superimpo ed upon a mild infection with PPLO produced a more serious disease in chickens

DIAGNOSIS

The presence of PPLO in the human body can be demonstrated only by cultivating the micro organisms in vitro as described in a previous section The characteristic appearances of the colonies on solid media and of the growth in liquid media are usually distinct enough to warrant the tentative placement of the micro organisms in the pleuropneumonia group In addition to their morphology, colonies of suspected PPLO may be further verified by staining in situ in agar blocks by the method of Dienes, or in petri dish cultures by means of diluted Dienes stain Cultures stained by Giemsa s or Wayson's method may be examined to differentiate further the organisms from bacteria To study further the etiologic role of a strain of PPLO isolated from a patient complement fixation tests may be performed with the patient's serum collected during the acute and the convalescent phases of the infection, as was done by Stokes (1955)

Serologic methods have been developed in avian infections A slide agglutination test employing a specially prepared antigen (Adler and Yamamoto 1956a) and a hemagglutination inhibition test have been employed for diagnostic work

TREATMENT

The sulfonamides, penicillin erythromycin polymyxin and bacitracin do not inhibit growth in vitro and are ineffective as therapeutic agents against the PPLO from man (Leberman et al Rubin et al, 1954) Streptomycin shows greater and more uniform inhibitory action in vitro than does dihydrostreptomycin (Leberman et al) That PPLO can develop drug resistance was demonstrated when a streptomycin sensitive strain became streptomycin resistant during therapy (Paine et al) when two strains developed resistance in vitro (Melen, 1952), and when a streptomycin resistant strain was discovered to be the cause of an outbreak of infectious sinusitis in turkeys (Fahey, 1957) Chlortetracycline and chloramphenicol inhibit the growth of some but by no means all strains in vitro Oxytetracycline and neomycin have an inhibitory action in vitro and oxytetracycline appears to be the drug of choice in the treatment of infections with PPLO (Leberman et al Robinson et al) Oxytetracycline and tetracycline also appear to be the drugs of choice in the treatment of infections caused by the avian strains of PPLO (Yamamoto and Adler, 1956)

Prior to the antibiotic era various gold compounds were found to be effective in preventing PPLO arthritis and encephalitis in mice and rats (Findlay et al) In patients with rheumatoid arthritis in whom the presence of PPLO could be demonstrated gold therapy brought about a remission of the clinical symptoms but the PPLO persisted Chlor

tetracycline therapy brought about the dis appearance of the organisms (Brown et al)

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Medical Mycology

GENERAL

The actinomycetes show a relationship not only to the single celled bacteria but also to the higher filamentous fungi or molds They exhibit branching like the higher forms but their size is nearer that of the Eubacteriales They are all slender organisms their vegetative structures being 1 micron or less in diameter Their methods of reproduction are essentially those of the bacteria, since they fragment readily into bacillary or coccoid elements or as found among the Streptomycetaceae they produce spores in the filaments as do the sporulating bacilli Also *Actinomyces* and *Nocardia* are sensitive to penicillin and sulfonamides respectively whereas the so called higher fungi are not affected by these agents Because of these characteristics the actinomycetes have been placed in the Schizomycetes in a position intermediate between the Eubacteriales or true bacteria and the Eumycetes or true fungi

The true fungi on the other hand are characterized by the formation of filaments or hyphae which branch and intertwine to form a dense mat of growth the mycelium which represents the colony of the fungus Produced on or from the mycelium are various types of reproductive bodies or spores This matlike growth made up of the mycelium with its spores constitutes an irregular rudimentary plant not differentiated into roots stems or leaves is called the thallus Plants characterized by this structure are classified in the *Phylum Thallophyta* The fungi constitute one of the two groups of plants in this Phylum The other group the algae are distinguished from the fungi by the presence of chlorophyll in the plant body whereas the fungi lack this material and are either saprophytic or parasitic

The relationship of the various groups to be found in the *Thallophytes* is shown in Table 51

TABLE 51

Thallophyta
Simple Vegetative Structures Not Differentiated into Roots Stems or Leaves

- Algae (Contain Chlorophyll)
- Fungi (Do Not Contain *Chlorophyll*)
  - I Pseudomycetes
    - 1 Schizomycetes—Bacteria
      - A Actinomyces
      - B Nocardia
    - 2 Myxomycetes—Slime Molds
  - II Euymycetes
    - 1 Phycomycetes—Water Molds
      - A Rhizopus
      - B Mucor
    - 2 Ascomycetes—Sac Fungi
      - A True Yeasts
      - B Highly Organized Forms Producing Ascospores
    - 3 Basidiomycetes—Club Fungi Mushrooms
    - 4 Fungi Imperfecti—Fungi Lacking Complete Life cycle i e Sexual Spores

Organisms known to be pathogenic for man and animals are found in only a few of the groups shown in Table 51 namely Schizomycetes which contains *Actinomyces* and *Nocardia* Phycomycetes which contains *Rhizopus* Ascomycetes which contains *Allescheria boydii* some species of *Aspergillus* and *Penicillium* known to have perfect stages and *Piedraia hortai* and the Fungi Imperfecti which contains all of the other known pathogens

Classification

With few exceptions (*Actinomyces Nocardia Cryptococcus Saccharomyces* and *Candida*) the fungi do not lend themselves to the use of bacteriologic technics for their identification They are all gram positive They do not ferment sugars do not reduce nitrates cannot be characterized by tests such as coagulation of serum or milk or the liquefaction of gelatin etc Therefore they are identified by their morphology macroscopically by the type of colony formation and microscopically by the type of spores produced on or from the mycelium Macroscopically there are 3 colony types yeast yeastlike and filamentous The yeast colony is soft and bacterial like and is composed of single celled budding forms (*Cryptococcus* and *Saccharomyces*) The yeastlike colony is also soft and bacterial like but is composed not only of single-celled budding forms on the surface of the medium but also of hyphae which penetrate the medium (*Candida* species) The filamentous colony presents the appearance of a typical mold and is composed of branching hyphae some of which penetrate the medium vegetative mycelium and some of which project from the surface aerial mycelium The aerial mycelium is referred to as a reproductive mycelium when spores are produced by the hyphae The type of spore produced and the method of its formation together with the type of colony provide the morphologic characteristics by which fungi are identified

The several types of spores produced by fungi may be divided into the two categories shown below

Spore Types

I Sexual Spores—produced as a result of nuclear fusion
1. *Ascospores*—spores produced in a sac the ascus found in the Ascomycetes
2. *Basidiospores*—spores produced from a club-shaped structure the basidium found in the Basidiomycetes
3. *Zygospores*—spores produced by fusion of two identical cell found in the Phycomycetes
4. *Oospores*—spores produced by fusion of two unlike cells found in the Phycomycetes

II Asexual Spores—produced in on or by the mycelium without nuclear fusion
1. *Thallospores*—spores produced by changes in the mycelium or thallus
   - A *Blastospores*—spores produced by budding *Saccharomyces Candida* and *Cryptococcus*
   - B *Chlamydospores*—spores produced by cells in the mycelium changing into thick walled resistant structures may be found in all fungi
   - C *Arthrospores*—spores produced by fragmentation of mycelium *Geotrichum* and *Coccidioides*
2. *True Conidia*—spores supported by a definite structure the conidiophore found among the Imperfecti
3. *Sporangiospores*—spores produced inside swollen structures sporangia on the ends of hyphae Phycomycetes

Examination of Fungi

Since the fungi can be identified only by the type and the arrangement of their spores preparations must be made that will allow examination of these structures The usual smear and staining method used for the bacteria are not applicable to filamentous cultures as they break up the hyphae disperse the spores and prevent proper examination A preliminary examination of a tube culture is possible by placing the tube on the microscope stage and using the low power objective on the top of the slant or along its edge

Slide cultures for continuous microscopic examination are made by various methods For example sterile slides and cover glasses under which inoculated warm agar is run support growth when placed in a damp chamber (sterile Petri dish with moist toweling or filter paper) Development of the fungus from the

edge of the agar may be followed microscopically In such preparations growth is undisturbed and the arrangement of the spores can be studied carefully

Routine examination of yeast or yeastlike cultures should be made by emulsifying some of the growth in a drop of water under a cover glass Filamentous fungi are examined by carefully teasing with needles some of the mycelium in a drop of mounting medium, i e lactophenol cotton blue or glycerine and eosin and covering the preparation with a cover glass

NOCARDIA (AEROBIC ACTINOMYCETES)

The aerobic actinomycetes of the genus *Nocardia* are free living in nature and several species may be introduced directly into wounds to cause localized actinomycotic mycetoma,' or *Nocardia asteroides* may be inhaled to cause a primary pulmonary disease with eventual metastases to any area of the body particularly to the subcutaneous tissues and the central nervous system

History

The first pathogenic aerobic actinomycete was described from *farcin du boeuf* in cattle by Nocard in 1888 and later was named *Nocardia farcinica* by Trevisan in 1889 The first pathogenic aerobic actinomycete found in human infection pseudotuberculosis with brain abscesses and meningitis was described by Eppinger in 1890 as *Cladothrix asteroides* and later named *Nocardia asteroides* by Blanchard in 1896 These two organisms are probably identical and the human species *N asteroides* has been retained because of its importance in medicine

Vincent (1894) described *Streptothrix madurae* as an etiologic agent of Madura foot Since this publication many species of aerobic actinomycetes have been described from the localized clinical entity actinomycotic mycetoma

Morphology and Variation

The *Nocardia* are gram positive partially acid fast or nonacid fast, branching filamentous organisms 1 μ or less in diameter Some species fragment readily into bacillary and coccoid elements while other species have a tendency to remain filamentous In tissues and in exudates from draining sinuses, in localized actinomycotic mycetoma they may appear as yellowish white, red or black dense, tangled mycelial granules with or without clubs at the periphery In spinal fluid, empyemic fluid, sputum and in pus from metastatic subcutaneous abscesses, in systemic nocardiosis only bacillary and branching forms may be seen Not infrequently the acid fast varieties may be mistaken for tubercle bacilli in stained sputum smears

Cultivation

Nocardia are aerobic organisms that grow readily at 37° C or room temperature on a variety of simple media such as beef infusion glucose agar, Sabouraud s glucose agar Czapek s agar, etc Colonies are slow growing, however and it is necessary to wait for 3 to 4 weeks before a typical appearance is obtained in the so called giant cultures' Such cultures show a marked variation in odor gross appearance pigment production and texture on different media and even on the same medium On solid media the colonies are usually glabrous wrinkled or granular and resemble those of some acid fast bacilli Occasional strains produce an aerial mycelium which gives the colony a chalky or powdery appearance, but on transfer, this character may be lost Pigmentation is produced best and is more constant on Czapek s agar and varies depending upon the species from cream to yellow orange ochraceous, and pink to coral or brick red The texture may be soft moist and mucilaginous or hard dry and granular On liquid media the species develop wrinkled surface pellicles with the medium remaining clear A comparison of the cultural appearance the pigment production the staining reaction and the morphology of 5 species of *Nocardia* pathogenic for man is presented in Table 52

In vitro studies of chemotherapeutic agents on species of *Nocardia* do not parallel in vivo studies so far as *N asteroides* is concerned Strauss et al (1951) found sodium sulfadiazine to be less effective than some of the antibiotics in vitro but to be more effective in vivo

Biochemical Reactions

A few of the biologic properties of the aerobic actinomycetes remain constant when different species of several strains of the same

TABLE 52 COMPARISON OF CULTURAL, MORPHOLOGIC AND STAINING REACTIONS OF SPECIES OF *Nocardia*

Species	Color of Granule	Acid Fast	Sabouraud's Glucose Agar	Czapek's Agar (Pigment)	Fragmentation of Mycelium
1 *Nocardia asteroides* (Eppinger) Blanchard 1896 Syn *Cladothrix asteroides* Eppinger 1890 *Streptothrix carnea* Rossi Doria 1891 *Streptothrix freeri* Musgrave & Clegg 1907 *Actinomyces gypsoides* Henrici & Gardner 1921 *Actinomyces ast* var *serratus* Satory Meyer & Meyer 1930 *Proactinomyces ast* var *crateriforme* Baldacci 1938 *Proactinomyces ast* var *decolor* Baldacci 1938	Yellowish white with or without clubs	+	Glabrous rarely chalky Moist soft *folded or wrinkled* and granular Yellow orange ochraceous red	Yellow to orange ochraceous	+
2 *Nocardia brasiliensis* (Lindenberg) Cast and Chalmers 1913 Syn *Discomyces brasiliensis* Lindenberg 1909 *Actinomyces mexicanus* Boyd & Crutchfield 1921	Yellowish white with or without clubs	+	Frequently chalky Folded cerebriform tenacious and dry Earthy odor Yellow orange ochraceous	Yellow to orange ochraceous	+
3 *Nocardia madurae* (Vincent) Blanchard 1896 Syn *Streptothrix madurae* Vincent 1894 *Nocardia indica* Chalmers & Christopherson 1916 *Actinomyces micetomae* Greco 1916 *Discomyces bahiensis* Piraja da Silva 1919	Yellowish white with or without clubs	—	Glabrous Moist soft wrinkled Cream colored	Cream colored at first later becoming pinkish to red	—
4 *Nocardia pelletieri* (Laveran) Pinoy 1912 Syn *Micrococcus pelletieri* Laveran 1906 *Nocardia africanus* Pijper & Pullinger 1927 *Nocardia Genesii* Froes 1930	Red with or without clubs	—	Small Glabrous heaped wrinkled Mucilaginous Coral pink to red	Coral red	—
5 *Nocardia paraguayensis* (Almeida) Conant 1947 Syn *Actinomyces paraguayensis* Almeida 1940	Black with clubs	—	Glabrous Soft white center Projecting border adherent darker	Dark cream to brownish	—

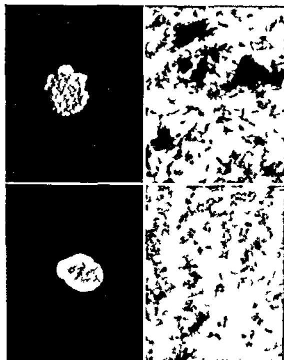


Fig 66 (*Top left*) *N asteroides* Colony on Sabouraud s glucose agar 12 days old (*Top right*) *N asteroides* from Sabouraud s agar Gram stain × 970 (*Bottom left*) *N brasiliensis* Colony on Sabouraud s glucose agar 17 days old (*Bottom right*) *N brasiliensis* from Sabouraud s agar Gram stain × 970

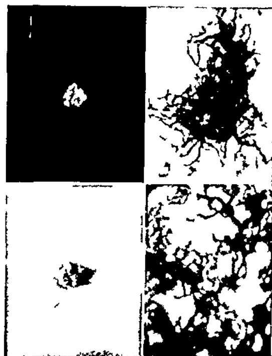


Fig 67 (*Top left*) *N madurae* Colony on Sabouraud s glucose agar 12 days old (*Top right*) *N madurae* from agar Gram stain × 970 (*Bottom left*) *N pelletieri* Colony on Sabouraud s glucose agar 12 days old (*Bottom right*) *N pelletieri* from Sabouraud s agar Gram stain × 970

species are tested they do not ferment carbohydrates and do not produce indol H S or NH_3 There is variation of reaction among strains studied by a single investigator and between single species studied by different investigators concerning coagulation of milk reaction in litmus milk reduction of nitrates and the ability to liquefy gelatin

Because of the wide variation of behavior both culturally and biologically among strains of pathogenic aerobic actinomycetes several different species have been described Gordon and Smith (1955) and Gordon and Mihm (1957) have attempted to resolve this problem by defining more exactly the generic differences of *Nocardia Streptomyces* and *Mycobacterium* as well as to differentiate species in the genus *Nocardia*

PATHOGENICITY

Nocardia asteroides is the only species showing pathogenic properties for laboratory animals However this species varies greatly in its ability to produce infection (Drake and Henrici 1943) Rabbits injected intravenously with a sufficiently large inoculum develop a generalized infection with the production of miliary abscesses throughout the entire body Intramuscular and subcutaneous injections result in local abscesses only Guinea pigs injected intraperitoneally usually show a diffuse peritonitis with abscess formation on the peritoneal surface Death results from a toxic effect of the inoculated material rather than from extensive invasion by the fungus Mice injected intraperitoneally with *N asteroides* suspended in 5 per cent hog gastric mucin die with sufficient regularity to be used for in vivo testing of chemotherapeutic agents (Strauss et al 1951) Injection into the yolk sac of embryonated hen s egg produces pro



Fig 68 (*Top*) Granules of N asteroides in subcutaneous tissue × 147 (*Bottom*) Granule of N pelletieri in subcutaneous tissue × 147

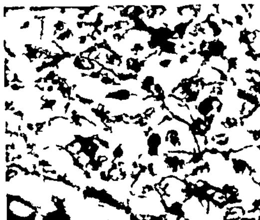


Fig 69 *Nocardia asteroides* Section of brain abscess Gram stain × 1300 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 41 Philadelphia Saunders)

lific growth of the organism and death of the embryo in 4 days (Brueck and Buddingh 1951)

Distribution

Nocardia are free living in nature have been described as air borne laboratory contaminants (Henrici and Gardner 1921) and have been isolated repeatedly from soil (Gordon and Hagan 1936 Habibi 1947 Emmons 1951) From this exogenous source the various species may be introduced into traumatized tissues to initiate localized chronic infection Therefore mycetoma is more prevalent in subtropical and tropical areas due to exposure of the body to the infectious organisms in the soil (Abbott 1956) However systemic nocardiosis has a world wide distribution and is not a rare primary pulmonary infection due to inhalation of infectious N *asteroides* (McQuown 1955) Spontaneous infections have been reported in dogs (Moss 1956) but animal to-man transmission is unknown

Pathogenesis

Nocardiosis is a chronic suppurative purulogranulomatous disease of the subcutaneous *tissues and the bones (Mycetoma) charac*terized by multiple tumefactions and draining sinuses from which granules (yellowish white red or black) are expressed in the pus or found in the tissues or a pseudotuberculous infection (systemic) of the lungs and the pleura with hematogenous spread throughout the body especially to the brain and the meninges in which filamentous bacillary or coccoid acid fast forms may be found in the sputum spinal fluid or pus from subcutaneous abscesses

Mycetoma of the extremities results in the clinical picture of Madura foot although

other subcutaneous tisues of the body also may become infected The characteristic le sion with pain swelling and sinus formation, and eventual clubbing and marked deformity of the infected member is developed only after months or years Infection spreads by exten sion through adjacent tissues with bone de struction multiple abscesses which rupture and with no systemic reaction unless second ary bacterial invasion is established Histo logically, sections of the sinus and abscess walls may show only a chronic inflammatory *reaction Further development of the acute* purulent abscess results in a surrounding layer of granulation tissue infiltrated with round cells and fat laden macrophages enclosed by a fibrous capsule However diagnosis depends on the presence of granules, surrounded by polymorphonuclear neutrophils centrally lo cated in the abscesses (Fig 68)

Systemic nocardiosis is caused by *N aste roides* and is chiefly pulmonary in origin

Occasionally headache nausea and vomit ing may suggest either brain tumor or brain abscess or the symptoms may be those of an infectious meningitis (tuberculous) with mini *mal or no findings in the lungs Symptoms* referable to a pulmonary infection include general malaise fever productive cough with sputum night sweats anorexia and loss of weight Roentgenograms of the lungs usually show a progressive infiltrative process which may lead to multiple cavity formation Hema togenous spread results in metastatic lesions throughout the body Histologically such le sions may be of a purulent nature containing centers composed of polymorphonuclear neu trophils and a few mycelial fragments which can be demonstrated only when the sections are stained by Gram s method (Fig 69)

Diagnosis

Lesions of the subcutaneous tissues produc ing the clinical picture of mycetoma should suggest infection by actinomycetes or infec tion by some of the higher fungi or molds (Maduromycosis) Pus from the draining sinuses scrapings from the sinus walls and biopsy sections should be examined for actino mycotic granules Fresh preparations of the pus and scrapings should be prepared by plac ing a drop of the material on a slide and covering the preparation with a cover glass Microscopically, the granules appear as amor phous lobulated masses surrounded by pus cells The mass is composed of delicate (1 μ in diameter) tangled hyphae which may or may not be terminated by sheaths (clubs) at the periphery Such granules should be crushed and Gram stained When examined the smear contains short branching forms bacillary and coccoid elements which are gram postive Cul tures are obtained by inoculating blood agar plates to be incubated at 37° C and Sabou raud s glucose agar slants to be incubated at room temperature, with crushed granules Anaerobic cultures also should be prepared since *Actinomyces bovis* produces a similar granule

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Cultures obtained from clinical materials can be tested for pathogenicity by intraperi toneal injection of guinea pigs with saline suspensions of the fungus by intraperitoneal injection of mice with 5 per cent hog gastric mucin or by yolk sac inoculation of the em bryonated hen s egg

Treatment

Although there is no specific treatment for Madura foot caused by the filamentous fungi *sulfadiazine therapy is effective for mycetoma* caused by the aerobic actinomycetes (Peters 1945 Calero 1947) The sulfonamides are effective in systemic nocardiosis if early diag nosis is made Sodium sulfadiazine alone or with *sulfamerazine to establish blood levels* of from 15 to 20 mg per cent should be given over a period of weeks or months (Peabody and Seabury, 1957)

Epidemiology

Pathogenic aerobic acid fast and non acid



Fig. 70. *Cryptococcus neoformans* 12 days on Sabouraud's glucose agar at room temperature.

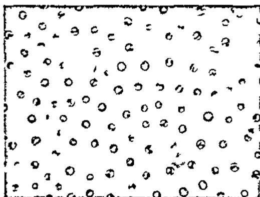


Fig. 71. *Cryptococcus neoformans*, round yeastlike cells with slight halo from Sabouraud's glucose agar at room temperature. × 490.

fast actinomycetes are free living in nature and cause disease by air borne contamination or by introduction into tissues through trauma. This view is substantiated by the study of systemic cases in which pulmonary infection is the rule, and of those cases demonstrating subcutaneous infection with no systemic reaction. There have been no reports of infection from man to man or from animal to man.

CRYPTOCOCCUS NEOFORMANS

Cryptococcus neoformans is a yeastlike, nonfermenting, nonsporulating, nonmycelial, budding fungus characterized by the development of a wide capsule both in tissue and in culture. It has a marked predilection for the central nervous system and produces a subacute or chronic infection of the meninges (Torula meningitis) or lesions simulating brain tumor or brain abscess but may also involve the skin, the lungs and other organs.

History

There are many early reports concerned with budding yeastlike fungi which, although given a variety of names and isolated from a variety of sources, are considered to be the same fungus, *Cryptococcus neoformans*.

A yeast, *Saccharomyces* sp., was described in Europe by Busse in 1894-1895 from a patient with a localized subperiosteal infection of the tibia who later died with multiple lesions of the skin and the viscera. Curtis in 1896 also described *S. tumefaciens* from a myxomatous tumor of the hip. In Italy, Sanfelice in 1895 isolated *S. neoformans* from the surface and the juice of peaches and compared it with *S. lithogenes* which he isolated from the lymph node of an ox with primary carcinoma of the liver; he found both to be pathogenic for laboratory animals. Later in 1898 he described *S. granulomatogenes* isolated from the lung of swine. Weis in 1902 described *Torula plimmer* from cancer of the breast, and *Torula sanfelice* from an adenocarcinoma of a human ovary. Frothingham in 1902 described a *Torula* sp. isolated from a tumor mass in the lung of a horse. However, Hansmann in 1905 was the first to report a yeast from the spinal fluid of a patient with suspected tuberculous meningitis.

In the United States, Stoddard and Cutler in 1916 reported 2 cases in man presenting signs of cerebral tumor which were caused by a budding fungus. The fungus isolated from one of these cases was compared with that obtained by Frothingham in 1902 and was named *Torula histolytica*. All these fungi are now considered to be identical.

Cultivation

Cryptococcus neoformans may be cultured at room temperature or at 37° C. on all common laboratory media. On Sabouraud's glucose agar at room temperature the colony is glistening, mucoid and tan to brown in color (Fig. 70). Microscopically, the cultures are best examined by emulsifying a portion of the growth in a drop of water or India ink under a cover glass. Such preparations reveal thick-walled, ovoid to spherical, budding cells 5 to 15 μ in diameter (Fig. 71), surrounded by a wide gelatinous capsule; no endospores are

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Epidemiology

Pathogenic aerobic acid fast and non acid



Fig 70 *Cryptococcus neoformans* 12 days on Sabouraud's glucose agar at room temperature

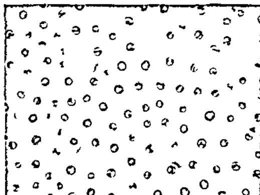


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Epidemiology

Pathogenic aerobic acid fast and non acid

Zimmerman 1956) However studies of antibody response in animals immunized with different strains have demonstrated capsular specificity of 3 serologic types designated A B and C (Evans 1949 Evans 1950 Evans and Kessell 1951) Also a capsular reaction has been reported with immune rabbit serum and *Cryptococcus* cells (Neill et al 1949 Evans et al 1956) Anticryptococcal sera has been shown to give serologic cross reactions with antigens from other fungi bacteria and with gum tragacanth (Evans et al 1953)

Soluble material in the spinal fluid the blood and the urine of a patient with cryptococcosis was found to precipitate in rabbit anticryptococcus serum (Neill et al 1951)

A skin test antigen has not been developed Nor are antigens or immune sera available as possible laboratory aids for the diagnosis of cryptococcosis Diagnosis is confirmed by the culture of *C neoformans* from the suspected case

Diagnosis

Sputum pus gelatinous exudates or sediment of centrifuged spinal fluid should be examined unstained by placing a small amount of the material on a slide and gently pressing to a thin film under a cover glass Also these materials should be mixed with a small amount of India ink and examined under a cover glass before the preparation dries The fungus appears as a thick walled spherical budding yeastlike cell 5 to 15 μ in diameter surrounded by a wide capsule (Fig 72) Specimens should be cultured on Sabouraud s glucose agar at room temperature and blood agar at 37 C

A positive urease test immediately distinguishes Cryptococcus sp from *Candida* sp and true yeasts Ability to grow at 37 C and pathogenicity for the mouse distinguishes *C neoformans* from other similar but nonpathogenic cryptococci

Treatment

A wide variety of drugs have been used to treat acute and chronic cryptococcosis (Littman and Zimmerman 1956 Wilson 1957) However the chronicity of the disease with known remissions extending over a period of 12 or more years makes it difficult to evaluate

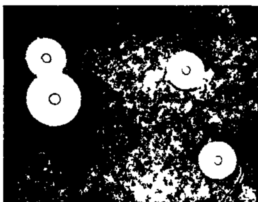


Fig 72 *Cryptococcus neoformans* India ink preparation of spinal fluid × 560

any therapeutic regimen The recent use of Amphotericin B in both acute and chronic infections has indicated that this antibiotic may prove of value in the treatment of cryptococcosis (Louria et al 1956 57)

Epidemiology

Contaminated soil is the source of infection for both animals and man Rarely primary cutaneous infection can be established by direct inoculation of the skin However the vast majority of cutaneous cryptococcosis is a manifestation of systemic infection with metastasis to the subcutaneous tissues following a primary pulmonary disease

No known cases of cross infection from animal to man or man to man have been reported

CANDIDA ALBICANS

Candida albicans is an oval budding yeast like fungus producing both blastospores and pseudomycelium in tissue and exudates and in culture at room temperature and at 37 C Its exact etiologic significance in any disease process is difficult to establish since it may frequently be found in the normal mouth and the intestinal tract or as a secondary contaminant in other recognized diseases

History

Langenbeck in 1839 demonstrated in thrush the presence of a yeastlike budding fungus which Robin in 1853 named *Oidium albicans* Zopf in 1890 renamed this fungus

produced and no mycelium is developed The composition of the capsular material has been investigated extensively and shown to contain a variety of polysaccharides (Benham 1956)

C neoformans is considered to be a non fermentive yeast in that gas is not produced in fermentation tests using various sugars However carbon and nitrogen assimilation tests are useful in distinguishing the single pathogenic from other closely related non pathogenic species *C neoformans* does not assimilate lactose but does glucose maltose, sucrose and galactose (Benham 1955) It does not reduce nitrate to nitrite but does hydrolyze urea (Seeliger 1956)

C neoformans is pathogenic for the mouse when injected intraperitoneally or intracerebrally

In summary *C neoformans* can be identified by the following criteria (1) positive urease test (2) growth at 37° C (3) assimilation tests with KNO_3 lactose glucose maltose sucrose and galactose and (4) demonstrated pathogenicity for the mouse

In vitro studies have shown *C neoformans* to be resistant to most antibiotics and chemotherapeutic agents However actidione prevents the growth of *C neoformans* in dilutions of 1 1 000 000 (Whiffen 1948)

Distribution

Cryptococcosis was first called European blastomycosis but the world wide distribution of reported cases soon discredited ideas of geographic limitation *C neoformans* exists as a saprophyte in nature and produces disease in a variety of animals (Barrow 1955) as well as man The fungus has been isolated repeatedly from soil (Emmons 1951) and is found in close association with pigeon nests (Emmons 1955 Kao and Schwarz 1957) There is no reported transmission of the disease from man to man or animal to man

Pathogenesis

Cryptococcosis is a subacute or chronic infection caused by *C neoformans* which may affect the skin the lungs or other tissues of the body with almost invariable meningeal involvement terminally Clinically cutaneous and systemic types of infection have been described The cutaneous may be primary or appear as a manifestation of an already established systemic infection Cutaneous lesions may appear as acneform pustules, punched out granulomatous ulcers, subcutaneous tumors or deep seated abscesses Many cutaneous cases progress to generalized infection with involvement of the lungs, the visceral organs and the central nervous system Systemic cryptococcosis may involve the brain meninges lungs liver, spleen pancreas thyroid and aorta In the majority of cases the central nervous system is the most frequently involved the lungs occasionally and other organs seldom Primary pulmonary infections resemble neoplasm or tuberculosis Brain infection may resemble an encephalitis acute or chronic meningitis of bacterial origin (especially tuberculous meningitis) brain tumor brain abscess central nervous system degeneration or central nervous system syphilis The spinal fluid pressure is increased globulin and albumin increased cell count high chlorides and sugar content low

There is frequent association (10 to 30%) of cryptococcosis and lymphoblastomatous diseases Concurrent fungus infection may be due to the debilitated state of the patient who acquires exogenous infection or to activation of an existing latent infection The latter hypothesis is borne out by the incidental finding at autopsy of small subpleural nodules containing *C neoformans* in patients whose death was due to a variety of unrelated conditions (Haugen and Baker 1954) The use of steroid therapy urethane mustard gas and folic acid antagonists may activate such latent infections by *C neoformans* and a variety of other fungi (Zimmerman and Rappaport 1954 *Keye and Magee 1956 Zimmerman 1957*)

The histology of the lesions in the brain varies greatly some sections show only a minimal reaction with surprising lack of inflammatory cells other sections may show pseudotubercles formed of giant cells epithelioid cells and lymphocytes The centers of such lesions may be necrotic or hyalinized Lesions in other tissues particularly the skin are typical granulomata

Immunity

Most attempts to demonstrate antibody response by the patient to *Cryptococcus neoformans* have produced negative or conflicting results, Cox and Tolhurst 1946 Littman and

Table 53 Differential Diagnosis of Species of *Candida**

	Pathogenic	Nonpathogenic					
	C albicans	*C tropicalis*	*C pseudotropicalis*	*C krusei*	*C parakrusei*	*C stellatoidea*	*C guilliermondi*
Sabouraud's agar	Creamy growth	Not characteristic	Not characteristic	Flat dry	Creamy	Creamy	Creamy growth
Sabouraud's broth	No surface growth	Narrow surface film with bubbles	No surface growth	Wide surface film	No surface growth	No surface growth	No surface growth
Blood agar	Medium sized dull gray colonies	Large gray colonies surrounded by mycelial fringe	Colonies small not characteristic	Colonies small irregularly shaped flat or heaped	Colonies small brilliant white	Colonies star shaped	Medium sized dull gray colonies
Corn meal agar	Branched treelike mycelium with chlamydospores	Mycelium well developed branched bearing numerous blastospores no chlamydospores	Mycelium poorly developed no chlamydospores	Crossed sticks mycelium no chlamydospores	Mycelium well developed no chlamydospores	Mycelium with large ball like clusters of blastospores	Mycelium well developed no chlamydospores
Glucose	AC	AG	AG	AG	AG[1]	AG	
Maltose	AG	AG				AC	
Sucrose	A	AG	AG				
Lactose			AG				

* Martin D S Jones C P Yao K F and Lee L F Jr 1937 A practical classification of the monilias Journal of Bacteriology *34* 99 129

[1] Occasionally acid only

Langeron and Guerra report acid and gas produced in glucose and sucrose when cultured at 5 C and held twenty days

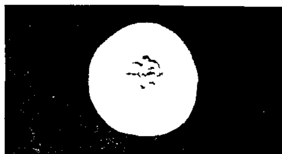


FIG 73 *Candida albicans* 20 days on Sabouraud's glucose agar at room temperature

Monilia albicans The rudimentary morphology of *M albicans* and the frequency with which similar yeastlike organisms have been isolated from infected and contaminated materials has made classification and identification of this group difficult (Conant 1940) The single genus *Candida* has been proposed for these yeastlike fungi and the number of species has been greatly reduced (Martin et al, 1937, Benham, 1957) *Candida albicans* has been considered to be the only pathogenic member of the genus However, other species are being isolated with increasing frequency from a variety of lesions in man

CULTIVATION

Candida albicans may be cultured on all common laboratory media both at room temperature and at 37 C On Sabouraud's glucose agar at room temperature, the colonies are cream colored and soft and have a distinct yeastlike odor (Fig 73) The surface growth is composed of oval, budding cells 2 5 x 4 to 6 μ while the submerged growth is composed of pseudomycelium This pseudomycelium in slide culture preparations is seen to consist of elongate undetached cells with clusters of blastospores distributed at the points of constriction (Fig 74) On corn meal agar, typical chlamydospores are produced (Fig 75) There is no surface growth on Sabouraud's glucose broth glucose and maltose are fermented with acid and gas sucrose with acid only, and lac



FIG 74 *Candida albicans* Pseudohyphae and clusters of blastospores on Sabouraud's glucose agar × 650 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 186 Philadelphia Saunders)



FIG 75 *Candida albicans* Chlamydospores on corn meal agar × 750 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 186 Philadelphia Saunders)

such infections may lead to meningitis Candidiasis also is recognized as a serious side effect of the intensive therapeutic use of chlortetracycline chloramphenicol or neomycin Lack of a competitive flora avitaminosis and actual enhancement of the growth of *C albicans* have been mentioned as possible factors influencing candidiasis during antibiotic therapy

Histologically sections of skin may show abscess formation or a chronic inflammatory reaction with giant cell formation Routine staining (H and E) may reveal tuberculoid granulomata with giant and epitheloid cells characteristic of tuberculosis However when stained by Gram s method such sections reveal the gram positive blastospores and the hyphal segments of *C albicans* in the centers of the tuberclelike structures and in areas of necrosis

Candidids occasionally accompany localized infections Such lesions are sterile and appear on the body as a result of sensitivity to the yeastlike fungi found in lesions elsewhere on the body

Although *C albicans* is considered to be the only truly pathogenic member of the genus 5 cases of mycotic endocarditis have been reported from which *C parakrusei* was cultured from 4 and *C guilliermondi* from 1

Immunity

The sera of patients with candidiasis will frequently show agglutination with saline suspensions of *C albicans* About 40 or 50 per cent of adults show a positive skin test to *C albicans* vaccine or oidiomycin Both these tests have doubtful diagnostic significance The constant finding of *Candida* species on the skin in the mouth and in the intestinal tract of apparently normal individuals could account for both agglutinins and sensitivity to these fungi It has been shown (Drake 1945) that by a slide agglutination technic 45 per cent of normal human sera agglutinate *C albicans* These findings indicate that agglutination tests have little value However hypersensitivity should be considered and properly evaluated when treating a case of candidiasis

Diagnosis

Sputum and materials from lesions in the mouth and in the vagina should be examined as fresh cover glass preparations and as gram stained smears Skin or nail scrapings should be placed in a drop of 10 per cent KOH under a cover glass and the preparation gently heated In fresh preparations *C albicans* appears as an oval budding yeastlike fungus 2 5 x 4 to 6 μ with occasional hyphal fragments 2 5 x 6 to 12 μ (Fig 76) *C albicans* appears as gram positive oval budding yeast like cells and gram positive elongated hyphal cells



Fig 76 *Candida albicans* budding yeast like cells in fresh preparation of sputum × 620

All materials should be cultured on Sabouraud s glucose agar at room temperature and at 37 C Clinical specimens should be streaked on Levine eosin methylene blue agar plates which are incubated at 37 C in a candle jar

Treatment

Oral and vaginal candidiasis may respond to alkaline mouth washes or alkaline douches respectively In both cases a 1 per cent gentian violet (in 10 to 20 % alcohol) used twice daily as a paint for 4 or 5 days has proved to be effective Sodium caprylate (Keeney 1946) and the methyl and propyl esters of parahydroxybenzoic acid (McVay and Sprunt 1951) also have been recommended for oral and vaginal candidiasis A propionate vaginal jelly (propion gel) has been reported as giving excellent results in vulvovaginitis (Alter et al 1947) Carbowax sulfur ointment (Hopkins et al 1952) has been effective in the treatment of intertriginous areas vaginal candidiasis and paronychia However chronic oral or vaginal candidiasis may resist all therapy including desensitization with oidiomycin

tose is not affected The common species of *Candida* can be differentiated by the technics of Martin et al (1937) Benham (1957) and Widra (1957) The different species are presented in Table 53

Candida albicans can be identified rapidly in clinical materials by its colony morphology on Levine eosin methylene blue agar (Weld 1953) With one exception *C stellatoidea* *C albicans* is the only species which produces mycelium in 14 to 18 hours on this medium when the streaked plates are incubated at 37° C in a candle jar

Distribution

Candida species are inhabitants of the normal mouth the intestinal tract and the vagina and may be cultured from these locations in 35 to 40 per cent of normal individuals Of this number about 15 to 20 per cent of the isolates have been identified as *C albicans* With rare exceptions however *C albicans* has not been isolated from normal skin *C albicans* is found frequently in the sputum of patients with proved nonmycotic pulmonary disease (tuberculosis and carcinoma) It is also found in quantity in the stools of patients with diarrheal symptoms due to other causes (sprue and pernicious anemia) Many species of *Candida* including *C albicans* have been isolated from a variety of animals (van Uden and Carmo Sousa 1957) and from exogenous sources in nature (Ajello 1956)

Of the several yeastlike fungi of the genus *Candida* only *C albicans* is pathogenic for laboratory animals Rabbits injected intravenously with 1 ml of a 1 per cent saline suspension die in 4 to 5 days with typical abscesses in the kidneys Also mice are susceptible to an intravenous injection of 0 1 ml of a 1 per cent saline suspension

Pathogenesis

Candida albicans may cause infections of the mucous membranes of the mouth (thrush) and the vagina (vaginitis or vulvovaginitis) infections of the skin particularly of the intertriginous areas (axillae inframammary inguinal intergluteal interdigital webs of the hands and the feet) infections of the nails (onychia and paronychia) and systemic infections (bronchopulmonary, or generalized infection of lungs lymph nodes liver, spleen and meninges)

Infection of the mouth (thrush) is encountered more frequently in children than in adults In children, it usually occurs following infection at birth from a mother with a vaginal infection (Taschdjion and Kozinn 1957) In adults infection with *C albicans* usually follows a debilitating illness Such lesions appear as extensive or scattered whitish patches which contain the blastospores and the pseudomycelium of the fungus Chronic oral lesions may last several years Occasionally the fungus spreads to the skin and the gastro intestinal tract to produce a generalized fatal moniliasis

Vulvovaginitis caused by infection of the vaginal mucosa and the vulva is a thrushlike infection characterized by irritation pruritus and a thin discharge

Infection of the skin usually occurs by autoinoculation of *C albicans* from the mouth or the intestinal tract Intertriginous lesions of the hand follow maceration of tissue by continued immersion in water Such lesions occur frequently in housewives waiters chefs bartenders fruit canners etc Intertrigo of the axillae and the intergluteal folds may become established because of obesity or diabetes Other intertriginous areas showing occasional infection are inframammary folds groin and interdigital webs of the toes Such lesions are characterized by erythematous exudative areas with well defined vesicopustular or papulosquamous borders

Infection of the nails (onychia and paronychia) is characterized by swelling at the nail bed which may be painful and resemble a pyogenic infection and by thickened transversely grooved nails

Infections of the lungs may cause a mild bronchopulmonary candidiasis with persistent cough and with sputum containing cellular debris and yeastlike cells Roentgenograms reveal slight to moderate peribronchial thickening and scattered rales may be heard at the base of the lungs More extensive pulmonary candidiasis may resemble miliary tuberculosis with cough fever dyspnea, chest pain hemoptysis and night sweats accompanied by signs of pleural thickening and consolidation

Systemic infection usually follows chronic refractory skin and oral lesions Infrequently

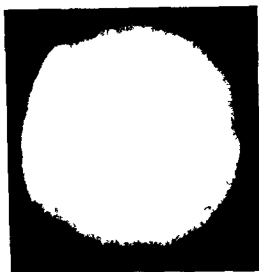


Fig 78 *Blastomyces dermatitidis* 21 days on Sabouraud's glucose agar at room temperature

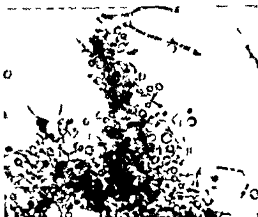


Fig 79 *Blastomyces dermatitidis* filamentous stage with conidia from Sabouraud's glucose agar culture at room temperature ×490

American blastomycosis namely *Glenospora gammeli Blastomycoides tulanensis Monosporium tulanense Endomyces capsulatus Endomyces dermatitidis* and *Glenospora brevis* A comparative study has shown them to be identical with Gilchrist's fungus *Blastomyces dermatitidis*

Cultivation

Blastomyces dermatitidis may be grown at room temperature or 37 C on all common laboratory media On blood agar or beef infusion glucose agar at 37 C the culture becomes wrinkled waxy and yeastlike in consistency (Fig 77) Microscopically it is composed of short broad 3 to 4 celled hyphal segments and budding yeastlike cells 8 to 20 μ in diameter similar to those seen in exudates or sections (Fig 77) On Sabouraud's glucose agar at room temperature the growth is at first smooth and yeastlike but quickly develops aerial projections and becomes prickly At this time a few budding cells may be found in such cultures but the majority of the growth has become filamentous A final overgrowth by white aerial mycelium which may turn brown with age establishes the completely filamentous stage of the fungus (Fig 78) Microscopically such a culture shows spherical to pyriform spores 5 to 8 μ in diameter attached directly to the hyphae or at the ends of short pedicels (Fig 79) This filamentous form can be converted to the yeastlike tissue phase by subculturing to blood agar and incubating at 37 C Temperature alone is responsible for the conversion of the mycelial to the yeast phase type of growth

Gelatin is not liquefied sugars are not fermented nor is milk affected

Lipids carbohydrates and proteins and a hemolysin have been isolated from the yeast phase cultures

Distribution

So far as is known *Blastomyces dermatitidis* is confined to the United States and Canada A case reported from Europe in an American soldier could have been acquired before leaving this country (Brody 1947) The incidence of blastomycosis on the North American continent seems to be related directly to an interest shown in the disease and an effort to find cases Many cases of spontaneous infection in dogs have been reported (Robbins 1954) and one in the horse (Benbrook et al 1948) Whereas most of the pathogenic fungi have been isolated from soil *B dermatitidis* has not been found as a saprophyte in nature

There have been no reports of man to man or animal to man transmission of the disease

Pathogenesis

Blastomycosis is a chronic granulomatous

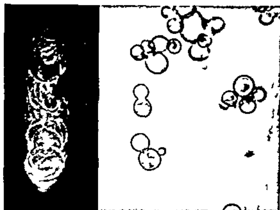


FIG 77 *Blastomyces dermatitidis* (*Left*) Yeastlike culture on blood agar 7 days at 37 C (*Right*) Yeastlike single budding cells from blood agar culture × 587

Intertriginous areas (hands or feet) should be treated with potassium permanganate soaks (1 4 000) 3 times daily, followed by a 1 per cent gentian violet (in 10 to 20% alcohol) paint or a 5 per cent ammoniated mercury ointment Other cutaneous areas may be treated similarly or with other materials (tincture of iodine salicylic benzoic acid ointment chrysarobin 5 to 10% etc) Onychia and paronychia may fail to respond to any of the above procedures Fractional x ray therapy may prove to be successful

Bronchopulmonary candidiasis is treated best with potassium iodide by mouth In a few cases ethyl iodide inhalation has been used successfully In the presence of hypersensitivity the patient should be desensitized with *C albicans* vaccine before iodides are administered (Conant et al 1954) Sodium iodide or gentian violet may be given intravenously in certain cases of pulmonary candidiasis Serum therapy (immune rabbit serum) of a case of widespread pulmonary candidiasis proved to be successful in a patient who had a negative skin test to *C albicans* vaccine a negative agglutination reaction to *C albicans* antigen but showed a positive Foshay type of reaction to an injection of immune serum previous to serum therapy (Hiatt and Martin 1946) However serum therapy can be expected to be successful only in those cases showing an excess of antigen and an absence of circulating antibody as demonstrated by a negative skin test and a negative agglutination reaction *Candidiasis following antibiotic therapy* may be partially controlled by adequate vitamin B complex intake

Nystatin (Brown and Hazen 1957) has been used successfully in a variety of cases of candidiasis (Benham 1957) Mycostechin (Nystatintetracycline compound) has been used to avoid the effects of treatment with a wide spectrum antibiotic

EPIDEMIOLOGY

Yeastlike fungi are found in the mouth in the intestinal tract in the vagina and on the skin of normal individuals The presence of a high percentage (40 to 50%) of positive skin reactions to Oidiomycin or to an autogenous vaccine indicates that individuals may become hypersensitive to these organisms or their products Autoinoculation from any of the sites mentioned can cause disease especially during intensive antibiotic therapy

CONTROL MEASURES

Patients with clinical candidiasis should be examined and treated for the presence of yeastlike fungi in the mouth or the intestinal tract to prevent autoinoculation

BLASTOMYCES DERMATITIDIS

Blastomyces dermatitidis is a spherical thick walled budding yeastlike fungus in tissue or exudates and in culture at 37 C In culture at room temperature it develops slowly as a typical moldlike filamentous fungus It produces a granulomatous infection of the skin and the internal organs very similar clinically and histologically to tuberculosis This disease is usually referred to as North American blastomycosis or Gilchrist s disease

HISTORY

Gilchrist, 1896 first described blastomycetic dermatitis a disease resembling tuberculosis from biopsy specimens which showed refractive double contoured budding cells in a section of skin A second case was reported by Gilchrist and Stokes 1896 from which they obtained a culture of the fungus which was named *Blastomyces dermatitidis* by Gilchrist and Stokes in 1898 Since these initial reports several fungi have been isolated from North

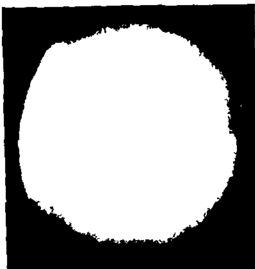


Fig 78 *Blastomyces dermatitidis* 21 days on Sabouraud s glucose agar at room temperature

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79) This filamentous form can be converted to the yeastlike tissue phase by subculturing to blood agar and incubating at 37 C Temperature alone is responsible for the conversion of the mycelial to the yeast phase type of growth

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So far as is known *Blastomyces dermatitidis* is confined to the United States and Canada A case reported from Europe in an American soldier could have been acquired before leaving this country (Brody 1947) The incidence of blastomycosis on the North American continent seems to be related directly to an interest shown in the disease and an effort to find cases Many cases of spontaneous infection in dogs have been reported (Robbins 1954) and one in the horse (Benbrook et al 1948) Whereas most of the pathogenic fungi have been isolated from soil *B dermatitidis* has not been found as a saprophyte in nature

There have been no reports of man to man or animal to man transmission of the disease

Pathogenesis

Blastomycosis is a chronic granulomatous

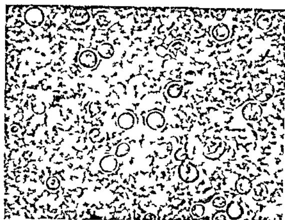


FIG 80 *Blastomyces dermatitidis* yeast like single budding cells in pus × 475 (Conant N F Smith D T Baker R D Callaway J L and Martin D S *Manual* of Clinical Mycology p 54 Philadelphia Saunders)

infection of the skin and the internal organs Primary cutaneous infection is rare and is characterized by an inflammation of the regional lymphatics which results in lymph adenopathy in association with the granulomatous or verrucous lesion (Schwarz and Baum 1951 Wilson et al 1955) Infection usually starts as a papulopustule which spreads peripherally showing a granulating base covered with a dirty pink exudate and a raised papilliform or verrucous border with miliary abscesses Spontaneous healing of the center produces scars of tissue paper thinness surrounded by the characteristic raised border

Primary pulmonary infection may be minimal or extensive and simulate tuberculosis or a malignancy With hematogenous dissemination, the subcutaneous tissues skin and bones are most commonly affected Skin lesions appear anywhere on the body as multiple subcutaneous gummalike lesions which rupture spontaneously freeing bloody pus The vertebrae and the ribs are the bones most frequently involved

Lesions are also found in the central nervous system, the liver the spleen and the kidneys where they are minimal and in the prostate The intestines are not affected (Kunkel et al 1954 Weed 1955 Cherniss and Waisbren 1956)

Two recent reports by Kunkel et al (1954) and Smith et al (1955) have shown that blastomycosis can occur as a primary pulmonary self limited disease similar to coccidioidomycosis histoplasmosis and tuberculosis

Histologically the lesions consist of numerous miliary abscesses containing polymorphonuclear cells, cellular debris and giant cells Also, tuberclelike lesions may be found which are indistinguishable from those seen in tuberculosis unless the fungus can be demonstrated

IMMUNITY

Complement fixing antibodies can be demonstrated in the serum of patients with extensive or progressive infection These antibodies denote extent of infection and signify a poor prognosis They cannot be demonstrated in patients with localized cutaneous lesions (Smith 1949 Martin 1953) Hypersensitivity to *Blastomyces* vaccine, carbohydrate and protein fractions of the yeast phase and to blastomycin can be demonstrated in most cases a delayed tuberculinlike reaction becomes positive in 24 to 48 hours

DIAGNOSIS

Crusts from verrucous lesions should be placed in a drop of 10 per cent KOH under a cover glass and examined microscopically after the preparation has been heated gently Sputum and pus from miliary abscesses at the border of cutaneous lesions or from subcutaneous lesions should be examined under a cover glass as untreated fresh preparations Spinal fluid and urine should be centrifuged and the sediment examined In all of these materials, *Blastomyces dermatitidis* appears as a thick walled, single budding yeastlike fungus 8 to 20 μ in diameter (Fig 80)

Culture materials on blood agar at 37 C and on Sabouraud s glucose agar at room temperature Heaped wrinkled isolated colonies which appear on blood agar should be transferred to slants for further development and identification

TREATMENT

The sulfonamides and the antibiotics seem to have no effect on the course of this disease Cutaneous lesions are treated best with iodides by mouth and x rays directed at the spreading border of the lesion Patients with systemic blastomycosis should receive supportive treat

ment a high caloric high vitamin diet with continued bed rest Iodides by mouth have been given with varying degrees of success In both the cutaneous and the systemic infections however, iodides should not be given until the patient has been tested for hypersensitivity to the fungus (Smith 1949) The patient should receive desensitizing injections of *Blastomyces* vaccine before iodides are started

The aromatic diamidines stilbamidine and dihydroxystilbamidine have been used successfully for the treatment of cutaneous and systemic blastomycosis (Curtis and Harrell 1952 Harrell et al 1955 Smith et al 1955) Also Amphotericin B has been used successfully in a few cases of blastomycosis which have failed to respond to the above drugs or have had periods of remission after adequate treatment with them

Pulmonary resections and lobectomies have been performed with some success (Schwarz and Baum 1951 Buechner et al 1953 White and Owen 1954)

Epidemiology

Blastomycosis is a disease of the North American continent—the United States and Canada The etiologic agent *B dermatitidis* probably exists in nature but unlike *Histoplasma Coccidioides* etc it has not been isolated from nature substrates Essentially the dog is the only known animal to have spontaneous blastomycosis However there has been no report of animal to man transmission of the disease

Blastomycosis is a primary pulmonary infection Few or many budding organisms can be demonstrated in the sputum and cultures are easily obtained However there have been no reports of man to man transmission of the disease (Chick et al 1956 Schwarz and Baum 1952 Schwarz and Goldman 1955)

Until recently only sporadic cases of blastomycosis have been reported In 1955 an epidemic of systemic blastomycosis involved 10 patients with the onset of the disease occurring within a 5 month period (Smith et al 1955 Harris et al 1957)

BLASTOMYCES BRASILIENSIS

Blastomyces brasiliensis is a large thick walled single and multiple budding yeastlike fungus in exudates and tissues and in culture at 37° C In culture at room temperature the fungus develops slowly as a heaped cerebriform glabrous smooth colony or as a slow growing filamentous colony with a short white aerial mycelium It produces a granulomatous infection of the mucous membranes of the mouth the lymph nodes and the internal organs The disease is known as South American blastomycosis Paracoccidioidal granuloma or Lutz Splendore Almeida s disease

History

Lutz in 1908 first reported pseudococcidioidal granuloma a localized disease of the mouth and regional lymphatics occurring in Brazil in which he described an organism thought to be closely related to *Coccidioides immitis* Carini in 1908 reported a second case of localized buccal infection and called the disease blastomycosis The first generalized infection was described as blastomycosis by Splendore in 1909 who later named the organism *Zymonema brasiliense* Haberfeld in 1919 renamed this fungus *Zymonema histosporocellularis* while Arantes in 1922 and Fonseca in 1928 1929 described it as *Coccidioides immitis* However Almeida in 1930 compared cultures of *C immitis* with cultures from South American blastomycosis and found them to be different He named the South American fungus *Paracoccidioides brasiliensis* and Moore in 1935 1938 added 2 new species *P cerebriformis* and *P tenuis* Conant and Howell in 1942 reduced these species to synonymy with *P brasiliensis* and placed the South American fungus in the genus *Blastomyces* as *B brasiliensis* A cheloidal type of blastomycosis reported from the Amazon region by Lobo in 1931 and said by Fonseca and Leao in 1940 to be caused by *Glenosporella loboi* has been regarded recently as a clinical variation of South American blastomycosis and the fungus reduced to synonymy with *B brasiliensis* (Lacaz 1956)

Cultivation

Blastomyces brasiliensis can be grown at room temperature or 37 C on all common laboratory media On blood agar or beef infusion glucose agar at 37° C the culture becomes smooth waxy and yeastlike (Fig 81) It is composed of numerous round multiple budding yeastlike cells 6 to 30 μ in diameter

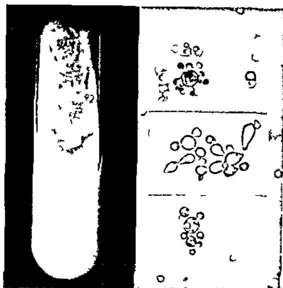


FIG 81 *Blastomyces brasiliensis* (*Left*) Yeastlike culture 12 days on beef infusion glucose agar at 37 C (*Right*) Multiple budding cells and moniliform cells from yeastlike culture at 37 C × 450



FIG 82 *Blastomyces brasiliensis* filamentous colony 28 days on Sabouraud s glucose agar at room temperature

with buds 1 5 to 5 μ in diameter scattered over the surface of the parent cell (Fig 81) Frequently single budding cells 8 to 14 μ in diameter are also seen These cells are identical with the yeastlike single budding forms seen in *Blastomyces dermatitidis* There can also be seen short 2 to 4 celled moniliform chains from the cells of which multiple buds are produced (Fig 81) On Sabouraud s glucose agar at room temperature *B brasiliensis* grows slowly (1 5 to 2 cm in diameter in 3 weeks) forming a compact culture which may be smooth at first but later develops short aerial mycelium white to light brown in color (Fig 82) These cultures are composed of hyphae with short broad thick walled cells 2 to 3 x 4 μ in size which easily dissociate Numerous intercalary and terminal chlamydospores and many noncharacteristic hyphal swellings are seen in the mycelium On the aerial mycelium some strains develop round to pyriform sessile conidia 3 to 5 μ in diameter

DISTRIBUTION

Blastomyces brasiliensis is confined to the South American continent where hundreds of cases have been reported from Brazil and sporadic cases from Argentina, Paraguay Uruguay Bolivia, Peru Ecuador, Colombia Venezuela Dutch and French Guiana (Floch and Saccharin, 1955 Lacaz 1956) Three cases have been reported from Costa Rica in Central America (Trejos and Romero 1953) and one from Mexico (Gonzales Ochoa and Esquivel 1950) One case has been described in a patient from the state of Oregon who had had previous residence in Venezuela (Perry et al, 1954) There have been no reports of spontaneous infections in animals in the endemic areas nor has the fungus been isolated from the soil

PATHOGENESIS

Blastomyces brasiliensis causes a chronic granulomatous infection of the mucous membranes of the mouth and the adjacent skin of the face the lymph nodes and the viscera The mouth seems to be the portal of entry where ulcerating vegetative papillomatous lesions on the buccal mucosa spread to the adjacent skin of the lips and the nose These lesions resemble those of yaws or mucocutaneous leishmaniasis The infection may spread to the regional lymphatics and hence to the axillary inguinal and other nodes Occasionally, direct infection of the lymph glands of the neck, without demonstrable buccal lesions produces massive glandular enlargement similar to that seen in Hodgkin s disease A chronic type of cutaneous blastomycosis resulting in cheloid lesions has been described from the region of the Amazon This clinical variation is referred to as Lobo s disease (Lobo 1933) In the visceral type of infection the intestines serve as the portal of entry Infection of the lymphoid tissue of the intestine with lymphatic drainage to the mesenteric nodes the spleen and the liver serves to establish massive visceral infection which simulates tuberculous peritonitis

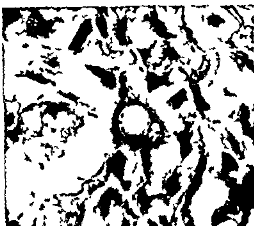


Fig 83 *Blastomyces brasiliensis* section of liver showing large thick walled cells with minute coccuslike buds × 10 5



Fig 84 *Histoplasma capsulatum* (Left) Yeastlike growth on blood agar at 37 C for 5 days (Right) Small budding yeast like cell from blood agar culture at 37 C × 570

Hodgkin s disease or carcinoma of the abdominal cavity While the intestinal tract in South American blastomycosis shows gross infection it should be noted that the intestinal tract is never involved in North American blastomycosis

Pulmonary infection usually follows lymphatic or hematogenous dissemination of the fungus from lesions elsewhere in the body but may occur as a primary pulmonary infection caused by inhalation of infectious materials The lesions cannot be distinguished from tuberculosis Boeck s sarcoid carcinoma etc

Histologically many areas show abscess formation with predominant polymorphonuclear infiltration other areas show focal lesions with necrotic and caseous centers surrounded by macrophages lymphocytes giant cells and fibroblasts The organisms appear in the tissue or giant cells as large round (10 to 60 μ in diameter) cells with small (1 to 5 μ) or large (10 to 30 μ) peripheral buds (Fig 83) This multiple budding is characteristic for *B brasiliensis* Frequent single budding forms 10 to 20 μ in diameter may be mistaken for *B dermatitidis*

Immunity

Positive complement fixation tests and positive intradermal skin tests have been reported in cases of South American blastomycosis As in North American blastomycosis a positive complement fixation test is indicative of a spreading infection (Lacaz 1945) It becomes negative after treatment with sulfonamides and is negative in minimal infections Skin tests with the filtrate of Sabouraud s broth in which several strains have been grown (paracoccidioidin) as well as with heat killed saline suspensions of the yeastlike phase of the fungus become positive in 24 to 48 hours Patients with South American blastomycosis frequently give a positive skin test and a positive serologic test with antigens from *Blastomyces dermatitidis* (Lacaz 1956)

Diagnosis

Pus and scrapings from the buccal mucosa and the skin lesions pus from fluctuant nodes and smears of biopsied nodes should be examined as fresh preparations under a cover glass Sputum in suspected pulmonary infections should also be examined In such materials *B brasiliensis* appears as large (10 to 60 μ in diameter) round multiple budding cells The buds may be large and few in number from the surface of the parent cell and measure 10 to 30 μ in diameter or they may be small and numerous and measure 1 to 5 μ in diameter

Materials should be cultured on blood agar at 37 C and on Sabouraud s glucose agar at room temperature Guinea pigs may be inoculated intratesticularly with the infected mate

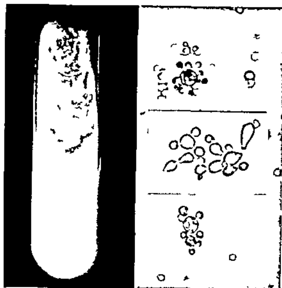


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smooth white to cream colored and resembles *Staphylococcus albus* (Fig 84) It is composed of small (2 to 4 μ) oval single budding cells (Fig 84) On Sabouraud s glucose agar *at room temperature it is cottony and white* at first but becomes buff to brown with age (Fig 85) Young cultures show branching septate hyphae bearing small (2 5 to 5 μ) *smooth round to pyriform spores on short* pedicles Older cultures contain large (8 to 20 μ) round to pyriform thick walled spores covered with fingerlike projections (Fig 86) These tuberculate spores *are* characteristic and diagnostic for *H capsulatum* The filamentous culture may be converted to yeastlike tissue phase by subculturing to blood agar slants which are incubated at 37 C Such yeastlike cultures may be maintained by subculturing to fresh blood agar slants every 4 to 5 days

Distribution

Histoplasmosis occurs throughout the world with areas of high endemicity as determined by histoplasmin skin testing programs (Edwards and Klaer 1956 Manos et al 1956) Areas of endemicity in the United States include the northeast central and south central states It is estimated that from 25 to 30 million people in the United States have had some form of the disease (Loosli 1957) *H capsulatum* has been isolated from the soil and spontaneous infection in a variety of animals has been reported (Emmons 1949 Emmons et al 1955)

Pathogenesis

Histoplasmosis is a primary pulmonary infection caused by inhalation of the highly infectious *H capsulatum* The respiratory infection may be subclinical mild or severe and simulate infection caused by any bacterial or viral agent In the vast majority of cases the infection is benign self limited and inapparent and revealed only by a positive skin test to histoplasmin

Acute pulmonary histoplasmosis is accompanied by fever cough malaise sweating and loss of weight In the lungs scattered miliary *multiple nodular* or parenchymal lesions may simulate tuberculosis Such lesions may undergo complete resolution but characteristically heal by calcification in 4 to 5 years (Bronsen and Schwarz 1957) A few of the pulmonary infections become chronic and progressive resulting in fibrocaseous lesions with cavity formation in the upper lobes (Sutliff et al 1953 Hawson et al 1956 Furcolow and Brasher 1956)

During the primary pulmonary infection the fungus may disseminate throughout the body by lymphatic or hematogenous spread to parasitize the reticuloendothelial system Symptomatic progressive disease apparently is *dependent on the resistance of the infected* individual Dissemination in the resistant individual has been demonstrated by a high rate of splenic calcification in known endemic areas of histoplasmosis (Schwarz et al 1955 Serviansky and Schwarz 1956) The nonresistant individual shows the *typical symptoms of* acute progressive histoplasmosis fever malaise sweats splenomegaly hepatomegaly leukopenia secondary anemia and emaciation Mucocutaneous lesions (skin tongue pharynx larynx) *are manifestations of systemic* infection with metastasis to the involved areas and occur frequently in chronic progressive infections (Baum et al 1957)

Histologically the lesions show a central *necrosis* with loss of tissue *and cellular structures* surrounded by granulomatous tissue The small 2 to 5 μ oval cells of the fungus appear in various phagocytic cells large *mononuclear or polymorphonuclear* cells of the blood *and* bone *marrow endothelial* wandering cells of tissues and fixed reticuloendothelial cells of the liver and the spleen

Immunity

Van Pernis *Benson and Hollinger (1941)* were the first to show that a patient and experimentally infected mice gave a positive skin test to filtrates of a broth in which *H capsulatum had been grown* A delayed tuberculin like reaction became positive *in 24 to 48 hours* Thousands of skin tests have been done with histoplasmin on people living in areas where there is known to be a high rate of nontuberculous *pulmonary calcification* as determined by negative tuberculin tests indicating a high resistance to the fungus

Serologic response to infection appears during the third or the fourth week Precipitins and complement fixing antibodies have been demonstrated (Salvin and Furcolow 1954 Loosli 1957)

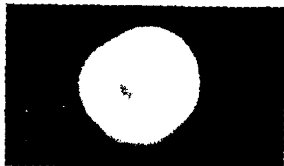


FIG 85 *Histoplasma capsulatum* 12 days on Sabouraud's glucose agar at room temperature



FIG 86 *Histoplasma capsulatum* typical tuberculate chlamydospore developed in the Sabouraud's glucose agar culture at room temperature × 556 (Smith D T Fungus diseases encountered in general hospital practice Am J Med 2 599 Fig 4B)

rials and cultures obtained after the development of lesions

Treatment

South American blastomycosis responds dramatically to the sulfonamides (Lacaz 1956) Sulfadiazine and sulfamerazine, when given over long periods of time have been found to be effective against the mucocutaneous lymphatic and visceral types of infection

Epidemiology

South American blastomycosis is a disease of rural communities workers in close association with farming and soil showing a high incidence of infection Males are infected more frequently than females (10 to 1) except among the Japanese where the ratio is not so great It is thought that the Japanese women who works in the fields with the men have an equal opportunity for infection Although it is felt that the fungus must have a saprophytic existence in nature it has not been found in the soil or on natural substrata nor have infections of animals been reported

Control Measures

There are no known effective control measures for South American blastomycosis

HISTOPLASMA CAPSULATUM

Histoplasma capsulatum is a small yeastlike, oval intracellular fungus in tissues and is yeastlike on blood agar slants at 37° C In culture at room temperature it is a typical moldlike filamentous fungus It is highly infectious it produces an acute benign self limited primary pulmonary infection and a chronic malignant progressive disseminated infection

History

Darling in 1906 first described an intracellular organism in the tissues from natives in the Canal Zone who had died of a disease similar to visceral leishmaniasis The organism was thought to be a protozoon closely related to *Leishmania donovani* and was named *Histoplasma capsulatum* Da Rocha Lima 1913 reported budding in these forms and called the organism *Cryptococcus* Hansmann and Schenken 1934 and De Monbreun 1934 were able to culture *H capsulatum* from their cases and proved it to be a filamentous fungus

Christie and Peterson (1945) and Palmer (1945 1946) reported the existence of inapparent or subclinical infections based on skin sensitivity to histoplasmin Until these reports histoplasmosis was considered to be a highly fatal fungus infection

Cultivation

Histoplasma capsulatum can be cultured on all common laboratory media On blood agar slants at 37° C, the growth is yeastlike

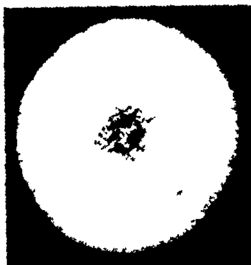


Fig 89 *Coccidioides immitis* 12 days on Sabouraud's glucose agar at room temperature



Fig 90 *Coccidioides immitis* hyphae developing characteristic arthrospore formation from Sabouraud's glucose agar at room temperature × 490

Rixford 1894 first described *from California* a protozoanlike organism in the tissue of a patient with lesions resembling tuberculosis cutis This case and a second occurring in *California were reported by* Rixford and Gilchrist *in 1896 Both patients were described* as having primary skin lesions with subsequent lymphatic dissemination resulting in death At *this time* the organism was named *Coccidioides immitis* and the disease became known as coccidioidal granuloma However Ophuls and Moffitt (1900) cultured the *organism* proving it to be a filamentous fungus Only coccidioidal granuloma was known until the *benign primary* type of infection was described by Gifford *in 1936*

Cultivation

Coccidioides immitis can be grown at room temperature on all common laboratory media On Sabouraud's glucose agar at room temperature the colony develops quickly with abundant aerial mycelium which is *cottony and* white at first but may become powdery and *buff* to brown with age (Fig 89) It shows *numerous arthrospores* which appear as deeply staining rectangular structures 2×4 *to* $5\ \mu$ separated by clear space along the course of *the hyphae (Fig 90)* In old cultures the hyphae *fragment freeing* the arthrospores, and the culture becomes friable and powdery Cultures should not be grown in Petri dishes because the powdery aerial *growth consisting* of numerous arthrospores is highly infectious The tissue phase (spherule formation) has *rarely been observed in artificial culture* (Converse 1956) However inoculation of arthrospores into the yolk sac of embryonated chick eggs results in a prolific growth of spherules *(Vogel and Conant 1952) Coccidioides immitis* liquefies gelatin *in 3 days milk is peptonized* and coagulated

Distribution

The arid southwestern United States includes *the known highly* endemic areas of coccidioidomycosis *The infection rate is high in* the southern San Joaquin Valley in California *and extends* across southern Nevada southwestern Utah, central and southern Arizona *southern* New Mexico through western Texas *along the Mexican border* A high rate of infection and clinical disease *occurs in the* northern states of Mexico in Honduras and Venezuela The Chaco region of Bolivia Paraguay *and* Argentina also is *an area of* high endemicity

Case reports from areas other than those *mentioned* above usually have a history of *previous* residence or travel through an en*demic area Sporadic* cases also may have be*come infected from contact with* contaminated materials (cotton wool etc) *exported from* an endemic area

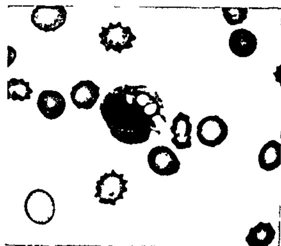


Fig 87 *Histoplasma capsulatum* peripheral blood smear showing *Histoplasma* in mononuclear cell × 1155

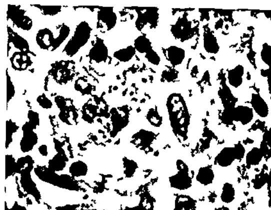


Fig 88 *Histoplasma capsulatum* section of gum showing intracellular bodies × 1012

Increased resistance of mice to experimental infection has been accomplished by immunization with dead yeast cells and cell wall material obtained from yeast cells (Salvin 1953 Salvin and Ribi 1955)

Diagnosis

Peripheral blood smears and sternal bone marrow smears should be stained and examined for intracellular small (2 to 5 μ) oval bodies in the polymorphonuclear and/or mononuclear cells (Fig 87) Lymph nodes skin and mucosal lesions should be biopsied and sections studied for the intracellular parasite (Fig 88) All such materials should be cultured on blood agar and Sabouraud s glucose agar at 37° C and room temperature respectively Contaminated materials such as sputum swabs from mucosal lesions and feces may be cultured on blood agar with the addition of 20 units of penicillin and 40 units of streptomycin per ml of medium Cultures may develop slowly and it may be necessary to hold them for at least 1 month before colonies appear

Treatment

A variety of drugs and antibiotics have been used without success (Loosli 1957) However Amphotericin B has recently given promising results

Residual granulomatous pulmonary lesions and cavitary pulmonary lesions have been removed by segmental resection or lobectomy (Binford 1955 Zimmerman 1954 Furcolow and Brasher, 1956 Hodgson et al 1951)

Epidemiology

H capsulatum exists as a saprophyte in the soil and both man and animals become infected by breathing in the highly infectious spores The disease is not transmitted from person to person or from animal to man Many epidemics have been reported involving a few or many individuals who had simultaneous exposure to the fungus in a storm cellar a cave a silo a chicken house etc (Lehan and Furcolow 1957)

COCCIDIOIDES IMMITIS

Coccidioides immitis is a spherical thick walled endospore filled organism in tissue or exudates and a fluffy white cottony fungus in culture at room temperature It produces a highly infectious disease (known as coccidioidomycosis) with an acute benign primary, self limited respiratory infection and a chronic malignant secondary progressive disseminated infection The secondary progressive disease is usually referred to as coccidioidal granuloma

History

Posadas 1892, and Wernicke 1892 first described from Argentina what is known now to be *Coccidioides* in the tissue of a patient with lesions similar to mycosis fungoides

be treated with cupric sulfate (0 05% final concentration), allowed to stand for 4 hours and then centrifuged sediment should be examined microscopically In all materials *C immitis* appears as a thick walled spherule, 10 to 80 μ in diameter filled with endospores (Fig 91) Infected materials should be cultured on blood agar at 37 C and on Sabouraud s glucose agar at room temperature A differential medium used by Smith is said to give excellent results for culturing *C immitis* from infected material (1% ammonium chloride 1% sodium acetate 0 8% tribasic potassium phosphate 0 04% cupric sulfate and 2% agar) Penicillin and streptomycin or chloramphenicol can be added to the medium to prevent bacterial contamination Mice should be inoculated intraperitoneally with infected materials or suspicious cultures Examine tissues or exudates from such animals for the characteristic spherules (Fig 92)



Fig 93 *Sporotrichum schenckii* 8 days on Sabouraud s glucose agar at room temperature



Fig 94 *Sporotrichum schenckii* delicate hyphae and conidiophores with terminal clusters of pyriform conidia from Sabouraud s glucose agar × 490

Treatment

In primary coccidioidomycosis the prognosis is excellent Treatment should be symptomatic with enforced bed rest until the temperature the sedimentation rate and the white count are normal

There is no specific treatment for the secondary progressive coccidioidal granuloma Symptomatic treatment with bed rest high vitamin high caloric diet may be helpful but prognosis is poor if multiple serologic tests show a rising titer in complement fixing antibodies

A variety of drugs have been used with little or no success in the treatment of progressive coccidioidomycosis Dihydroxystilbamidine has given promising results in a few cases (Snapper et al 1955) Amphotericin B should be given adequate clinical trial because of its reported effectiveness in other fungus diseases

Localized residual pulmonary coccidioidal lesions (coccidioidomata and cavities) require surgical management either lobectomy or segmental resection (Winn 1957)

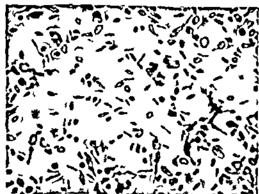


Fig 95 *Sporotrichum schenckii* fusiform cells from cystine agar culture at 37 C × 580 (Smith D T Fungus diseases encountered in general hospital practice Am J Med 2 602 Fig 7B)

Epidemiology

Coccidioidomycosis is a dust born disease of the arid regions of the western and the southwestern parts of the United States In

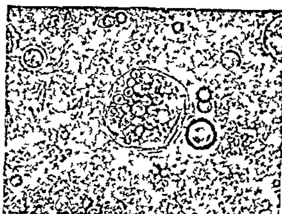


FIG 91 *Coccidioides immitis* large thick walled endospore filled spherule and smaller immature spherules in pus × 490



FIG 92 *Coccidioides immitis* section of *lung showing mature and immature sphe* rules × 490

PATHOGENESIS

C immitis causes an acute benign respiratory disease which is usually self limited in the white skinned race but has a tendency to develop into a progressive malignant disseminated highly fatal disease in the dark skinned races Primary infection usually takes place in the *respiratory tract (rarely skin abrasions)* by breathing in dust containing infectious material (Wilson et al , 1953 Trimble and Doucette 1956) *The infection may be subclinical* or after an incubation period of 8 to 14 days the symptoms may be those of bronchial pneumonia or flu chills fever malaise anorexia, cough pleurisy, headache backache and night sweats About 2 to 5 per cent of such cases develop hypersensitivity which becomes evident after 5 to 14 days as typical erythema nodosum or erythema multiforme with skin lesions of from 1 to 4 weeks duration This form of the infection is not fatal and is known as San Joaquin Fever Valley Fever and Desert Rheumatism when associated with allergic manifestations

Dissemination at the time of the primary infection gives symptoms similar to those of tuberculosis Lesions may appear anywhere in the body lungs, larynx lymph nodes bones, joints central nervous system etc This form of the infection is highly fatal and is known as coccidioidal granuloma

Histologically the lesions are granulomata with tuberculelike formation in which the typical endosporulating spherule must be seen to distinguish them from tuberculous lesions Recently Puckett (1954) and Marshall et al (1955) have described the unusual occurrence of fungus hyphae accompanying the spherule stage of *C immitis* in tissues

IMMUNITY

Complement fixing antibodies and precipitins can be demonstrated in the serum of patients infected with *C immitis* (Smith et al , 1956) The complement fixing antibodies in high titer have the same significance as in blastomycosis i e indicate spreading infection and poor prognosis Hypersensitivity to the fungus can be demonstrated by the coccidioidin skin test (Smith et al 1948) A positive test usually develops in from 3 to 21 days following infection and gives a delayed tuberculinlike reaction in from 24 to 48 hours After a positive skin test to coccidioidin has been acquired the individual is immune to exogenous reinfection (Smith, 1943)

Immunization of mice with killed arthrospores of *C immitis* has extended the survival rate of experimentally infected animals (Friedman and Smith 1956)

DIAGNOSIS

Pus and sputum should be examined as untreated, fresh preparations under a cover glass Pleural fluid and gastric contents should be centrifuged and the sediment examined similarly Sputum and gastric contents also may

be treated with cupric sulfate (0 05% final concentration) allowed to stand for 4 hours and then centrifuged sediment should be examined microscopically In all materials *C immitis* appears as a thick walled spherule *10 to 80 μ in diameter filled with endospores* (Fig 91) Infected materials should be cultured on blood agar at 37 C and on Sabouraud s glucose agar at room temperature A differential medium used by Smith is said to *give excellent results for culturing C immitis* from infected materials (1% ammonium chloride 1% odium acetate 0 8% tribasic potassium phosphate 0 04% cupric sulfate and 2% agar) Penicillin and streptomycin or chloramphenicol can be added to the medium to prevent bacterial contamination Mice should be inoculated intraperitoneally with infected materials or suspicious cultures Examine tissues or exudates from such animals for the characteristic pherules (Fig 92)



Fig 93 *Sporotrichum schenckii* 8 days on Sabouraud s glucose agar at room temperature



Fig 94 *Sporotrichum schenckii* delicate hyphae and conidiophores with *terminal* clusters of pyriform conidia from Sabouraud s glucose agar × 490

Treatment

In primary coccidioidomycosis *the prognosis* is excellent Treatment should be symptomatic with enforced bed rest until the temperature the sedimentation rate and the white *count are normal*

There is no specific treatment *for the secondary* progressive coccidioidal granuloma Symptomatic treatment with bed rest high vitamin high caloric diet may be helpful but *prognosis is poor if multiple serologic tests* show a rising titer in complement fixing antibodies

A variety of drugs have been used with little or no success in the treatment of progressive *coccidioidomycosis Dihydroxystilbamidine* has given promising results in a few cases (Snapper et al 1955) Amphotericin B should be given adequate clinical trial because of its reported effectiveness in other fungus diseases

Localized residual pulmonary coccidioidal lesions (coccidioidomata and cavities) require surgical management either lobectomy or seg*mental resection* (Winn 1957)

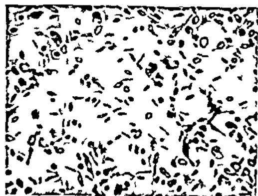


Fig 95 *Sporotrichum schenckii* fusiform cells from cystine agar culture at 37 C × 580 (Smith D T Fungus di eases encountered in general hospital practice Am J Med ‸ 60? Fig 7B)

Epidemiology

Coccidioidomycosis is a *dust born disease* of the arid regions of the western and the southwestern parts of the United States In

habitants in these areas show a high incidence of infection as demonstrated by a positive coccidioidin skin test The infection rate of susceptibles (newcomers) follows closely a seasonal variation most cases arising in the dry summer and autumn months when rainfall is lowest and dust more prevalent *C immitis* has been cultured numerous times from the soil and animals as well as man become in fected There is no person to person or animal to man transmission of the disease

Control Measures

Studies carried out in conjunction with the Army Air Forces in 4 air fields in the San Joaquin Valley Calif during the period from 1941 to 1946 showed that dust control of such areas reduced infection rates from one half to two thirds in nonimmune susceptibles Paving roads and runways planting lawns and using refined oil on athletic areas proved to be effec tive control measures (Smith et al 1946)

SPOROTRICHUM SCHENCKII

Sporotrichum schenckii is a single celled fusiform or round budding yeastlike fungus *in the exudates or tissues of animals (rarely* seen in exudates or tissues of man) and in culture on cystine containing medium at 37 C Cultures at room temperature and on noncystine containing media at 37° C are leathery filamentous and usually brownish to black in color *S schenckii* frequently causes a localized lymphangitic infection and rarely a systemic infection in man

History

Schenck, 1898 first cultured and described this fungus from a patient in the United States showing refractory subcutaneous ab scesses Hektoen and Perkins 1900 in de scribing the second case named the fungus *Sporothrix schenckii* The disease was de scribed by de Beurmann and Ramond 1903 in France and the fungus was named *S beurmanni* by Matruchot and Ramond in 1905 This species and several others described from time to time are now thought to be variants of Schenck s fungus and are usually considered as synonyms of *S schenckii*

Cultivation

Sporotrichum schenckii can be grown at room temperature or at 37 C on all common laboratory media On Sabouraud s glucose agar at room temperature the colonies ap pear as small, white growths lacking aerial mycelium (Fig 93) As growth increases, the surface of the colony becomes folded and leathery *the color may vary from white to* tan or brown to black, depending upon the medium and the individual strain Microscopi cally, the colony is composed of delicate branching septate hyphae 1 5 to 2 μ in diam eter Pyriform conidia, 2 to 4 by 2 to 6 μ in size are borne at the ends of lateral branches (conidiophores) in characteristic clusters (Fig 94) In some strains these conidia are al o borne directly from the hyphae On cystine agar at 37° C the growth remains soft and yeastlike and is composed of fusiform bodies (tissue phase) and short mycelial fragments (Fig 95)

The biochemical activities of *S schenckii* vary with individual strains (Lurie 1950) Gelatine may be liquefied but only after many days sugars if fermented show acid only Milk may or may not be coagulated

Distribution

Sporotrichosis is world wide in distribution In the United States it is thought that endemic *areas include* Nebraska Wisconsin Kansas the Dakotas and Missouri Although the larger number of cases have been reported from the Mississippi Valley it is probable that numer ous cases also have been recognized elsewhere but have not been reported

The fungus is widely distributed in nature and may be recovered from plants animals and a variety of contaminated objects

Pathogenesis

Sporotrichosis is a subacute or chronic granulomatous infection which follows intro duction of the fungus by trauma The initial lesion usually appears on exposed areas, par ticularly the extremities and develops as a single lesion (pustule ulcer abscess or chan cre) which fails to heal under ordinary treat ment With invasion of the regional lym phatics, the characteristic picture of localized lymphangitic sporotrichosis develops ascend ing chronic lymphangitis with cordlike thick ening of the lymph vessels and multiple sub cutaneous abscesses along the course of the infected lymphatics These abscesses are gum malike and may or may not rupture spontane ously The epitrochlear and axillary nodes are

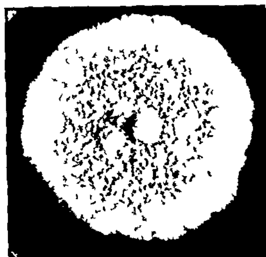


FIG 96 *Monosporium apiospermum* 15 days on Sabouraud's glucose agar at room temperature



FIG 9 *Monosporium apiospermum* conidiophores terminated by a single conidium × 230 (Conant N F Smith D T Baker R D Callaway J L and Martin D S Manual of Clinical Mycology p 248 Philadelphia Saunders)

usually not enlarged and systemic reactions are rare The ulcerating nonhealing primary chancrelike lesion with lymphatic involvement suggests tularemia or syphilis This localized lymphangitic type of infection is most prevalent in the United States Single lesions about the eyes on the face the neck and on the body may not be accompanied by regional lymphangitis and create a difficult therapeutic problem unless sporotrichosis is considered (Cipollaro and Singer 1952 McGrath and Singer 1952 Singer and Muncie 1952) Other clinical manifestations have been described by Beurmann and Gougerot (1912) varying from a localized lymphangitis to a widely disseminated subcutaneous or systemic gummatous sporotrichosis An occasional disseminated case of sporotrichosis is seen in the United States (Collins 1947)

Histologically the lesions may show only a nonspecific chronic inflammatory process or become granulomatous with lymphocytic infiltration plasma cells giant cells and fibrosis The fusiform bodies are rarely seen in such sections or in the pus from the lesions Therefore diagnosis by direct examination of these materials is doubtful or impossible

Immunity

Agglutinins precipitins and complement fixing antibodies can be demonstrated in the serum of patients infected with *S schenckii* (Norden 1950) A high degree of sensitivity beginning after about 5 days can be demonstrated by using heat killed saline suspensions of the fungus or a carbohydrate fraction (Gonzales et al 1947) as the skin test material A delayed tuberculinlike reaction manifests itself in 24 to 48 hours

Diagnosis

Positive cultures are the best means of diagnosis The fusiform bodies that are found in experimentally infected animals are discovered only rarely in materials from human lesions Pus from unruptured nodules swabs scrapings and biopsies of ulcerated lesions should be obtained and cultured both at room and incubator temperatures The characteristic colony with its typical spore formation allows identification of *S schenckii*

Treatment

Sporotrichosis responds readily to iodides administered orally over long periods Recurrences can be avoided by extending the treatment 1 or 2 months after apparent cure An occasional resistant case may respond to stilbamidine (Harrell et al 1954)

Epidemiology

Sporotrichosis has been said (Foerster 1926) to be an occupational disease of horti

culturists for many infections could be traced to injuries by barberry thorns That the infection could assume a major role as an industrial disease was pointed out by du Toit (1942) in his report of 650 cases among miners and native workers in the gold mines of the Witwatersrand in South Africa This epidemic in the mines finally totaled 2 825 cases The fungus was isolated from the mine timbers and from the dust and infection usually followed injury by machines or rock and scratches by wire Individuals in contact with infected rats dogs horses and mules may become infected

MONOSPORIUM APIOSPERMUM

Monosporium apiospermum is a fungus which appears as a large macroscopic grain or granule, white to light yellow in color made up of wide septate hyphae in tissue or exudates from draining sinuses In culture it is a gray to light buff filamentous fungus which reproduces characteristically by single spores from the ends of conidiophores This fungus and many others (several genera and species) produce the disease known as maduromycosis or Madura foot

History

The discovery of maduromycosis or mycetoma in India and the early history of this type of fungus infection has been summarized by Gammel (1927) The confusion relative to etiologic agents was clarified by Chalmers and Archibald (1916) when they proposed that the disease mycetoma should be classified into two categories according to the type of fungus causing the infection (1) actinomycotic mycetoma caused by species of *Nocardia* and (2) maduromycosis caused by the higher filamentous fungi This classification has been adopted by most investigators

M apiospermum has been isolated from numerous cases of maduromycosis in the United States and *Allescheria boydii* isolated by Boyd and Crutchfield in 1921 has been shown by Emmons (1944) to be the perfect ascomycetous stage of *M apiospermum* Other fungi reported as etiologic agents of maduromycosis in the United States include *Madurella ikedai* *Madurella lackawana* *Madurella mycetomi* *Madurella grisea* *Phialophora jeanselmei* and *Cephalosporium granulomatis* (Neuhauser 1955 Weidman and Kligman 1945)

Cultivation

M apiospermum can be cultured on all common laboratory media On Sabouraud s glucose agar at room temperature, the colony develops quickly as a white cottony aerial growth which becomes grayish to buff in color (Fig 96) A black pigment is usually produced in the agar on the reverse side of the colony

Microscopically single ovoid to clavate conidia, 5 to 7 x 8 to 10 μ in size with truncate bases are seen on the ends of conidiophores of various lengths (Fig 97) Coremia (bundles of hyphae) may also be seen with conidia terminating the individual hyphae coming from the bundle In some strains conidia are produced laterally from the hyphae and occasionally in small clusters However the usual method of spore production is the *development of single conidia from the ends* of conidiophores The ascomycetous phase of *M apiospermum* is *Allescheria boydii* isolated by Boyd and Crutchfield (1921) In this culture, the cleistothecia (closed perithecia) 50 to 200 μ in diameter are thin walled brownish structures containing subglobose asci in each of which are seen 8 elliptical faintly brown walled ascospores 4 to 4 5 x 7 to 7 5 μ in size

Distribution

Maduromycosis is endemic in India and the majority of the cases reported elsewhere in the world have been found in the tropics Summaries of the distribution of this infection and lists of the multiplicity of fungi which have been isolated are contained in reports by Mackinnon (1954) Lacaz and Fava Netto (1954) and Abbott (1956)

In the United States *M apiospermum* has *been reported from cases of* maduromycosis in Texas Massachusetts, Georgia (2) Maryland North Carolina and Pennsylvania Outside of the United States *M apiospermum* has been reported from cases of maduromycosis in Canada the Virgin Islands Paraguay Argentina Brazil, Algeria and Italy Recent isolations of *M apiospermum* (*A boydii*) from soil have given final proof of its saprophytic existence in nature (Ajello 1952)

Maduromycosis has recently been reported in animals (Seibold 1955 Bridges 1957)

Pathogenesis

Maduromycosis is a chronic slowly progressive unilateral infection of the subcutaneous tissues caused by the introduction of one of several different filamentous fungi by trauma The majority of cases have occurred on the foot but occasional infections of the leg and the hand have been described Infection usually follows an injury which heals and after varying periods of time becomes noticeable by the formation of papules deep-seated nodules or abscesses which rupture to form multiple draining sinuses In some instances the infection begins with swelling and pain and the subsequent development of indurated areas which become open fistulae from which drains serosanguineous fluid containing the characteristic granules With extension of the infection to the fascia muscles and bone and the development of dense fibrous tissue the foot becomes swollen club-shaped and markedly deformed Osteomyelitis of the bones of the foot may cause extensive fusion of these structures resulting in stiffness and loss of motion There is usually no systemic reaction to the infection and little if any pain

Histologically the lesions are similar to those of actinomycosis Abscess formation is prominent Granules situated in the pus may be surrounded by a granulation tissue composed of polymorphonuclear cells plasma cells lymphocytes eosinophils and macrophages Giant cells may or may not be present The granules are rounded to lobulated structures composed of wide septate hyphae with chlamydospores around the periphery

Immunity

Agglutinins have been demonstrated in the sera of several patients with maduromycosis due to *M apiospermum* In a case of chronic infection precipitinins complement fixing antibodies and skin sensitivity could be demonstrated (Seeliger 1956)

Diagnosis

Materials from fistulae and draining sinuses and biopsy specimens should be examined for granules These are oval lobulated 0 5 to 2 mm in diameter white to light yellow granules when *M apiospermum* is the infecting fungus Other fungi *Madurella sp* produce



Fig 98 *Monosporium apiospermum* section from subcutaneous tissue showing granule (Smith D T Fungus diseases encountered in general hospital practice Am J Med 60 Fig 8)

black granules Microscopically the maduromycotic type of granule is composed of wide branching septate hyphae 2 to 4 μ in diameter with numerous chlamydospores (Fig 98) The actinomycotic type of granule on the other hand is composed of fine delicate nonseptate hyphae 1 μ or less in diameter These two types of granules should be distinguished in microscopic preparation for a correct diagnosis

Granules should be washed in sterile saline or broth crushed and streaked on Sabouraud s glucose agar or beef infusion glucose agar to which antibiotics have been added to avoid bacterial contamination

Treatment

There is no specific therapy for maduromycosis In one case caused by a *Cephalosporium* of 2 years duration in which the infection was confined to the skin and subcutaneous tissue Twining et al (1946) reported apparent cure with penicillin Neuhauser (1955) reported promising therapeutic results with diaminodiphenylsulfone in a patient infected with *Madurella grisea* However maduromycosis is more effectively treated by surgical management supplemented by sulfonamide or anti



FIG 99 *Hormodendrum pedrosoi* (*Left*) Twenty one days on Sabouraud s glucose agar at room temperature (*center*) *Hormodendrum* type of conidiophore × 440 (*right*) *Acrotheca* type of conidiophore × 440 (*Right* and *center* Conant N F Martin D S Smith D T Baker R D and Callaway J L Manual of Clinical Mycology p 100 Philadelphia Saunders)



FIG 100 *Hormodendrum compactum* (*Left*) Thirty eight days on Sabouraud s glucose agar at room temperature (*Right*) Conidiophore with compact spore head × 470

biotic therapy to control secondary bacterial infection Early diagnosis and excision of the affected localized area should be curative

EPIDEMIOLOGY

Maduromycosis is a disease of the tropics and the subtropics with occasional cases reported in the temperate zone It is a disease of the exposed parts of the body particularly of the feet Most of the fungi belong to genera known to contain saprophytic species which may be cultured from the soil Injuries to persons going without shoes predisposes to infection Men are infected more commonly than women all races are susceptible

A boydii (*M apiospermum*) has been isolated from the spinal fluid in a case of meningitis (Benham and Georg 1948) and from the sputum in a case of chronic pulmonary infection (Creitz and Harris 1955)

CONTROL MEASURES

From the nature of the infection the type of fungi involved and the almost inevitable history of injury to bare feet it would seem that wearing shoes would be a practical control measure

HORMODENDRUM PEDROSOI

Hormodendrum pedrosoi is a small round thick walled dark brown body found in crusts exudates and tissue where it reproduces by splitting In culture it is a dark green to brown filamentous fungus which reproduces by a variety of spore forms This fungus with several others (*H compactum, H dermatitidis H carrionii* and *Phialophora verrucosa*) cause the disease known as chromoblastomycosis

HISTORY

Pedroso 1911 in Brazil observed dark bodies in the tissue from a patient with verrucous skin lesions but failed to identify the fungus which he isolated Later Pedroso and Gomes 1920 reported 4 Brazilian cases including Pedroso s original patient and named the etiologic agent *Phialophora verrucosa* This identification was based on an earlier report by Lane 1915 and Medlar 1915 in which they described *P verrucosa* a fungus isolated from nodular lesions on the buttocks of an Italian in Boston in whose tissue they also had observed dark brown splitting bodies Brumpt 1922 restudied the South American fungus and named it *H pedrosoi* Terra et al 1922 called the disease chromoblastomycosis Carrion 1935 described *H compactum* from Puerto Rico Kano 1937 described *H* (*Hormiscium*) *dermatitidis* from a case in Japan Trejos 1954 described *H carrionii* from several isolates from patients in South Africa Venezuela and Australia

While *P verrucosa* and *H compactum* have remained constant in their morphology *H pedrosoi* has varied considerably and has presented a real problem in classification Since *H pedrosoi* has been isolated from the majority of the cases of chromoblastomycosis this has led to numerous attempts to reclassify the fungus (Conant et al 1954)

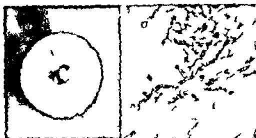


Fig 101 *Phialophora verrucosa* (*Left*) Twenty-eight days on Sabouraud's glucose agar at room temperature (*right*) typical conidiophore × 410

Cultivation

H pedrosoi can be cultured on all common laboratory media On Sabouraud's glucose agar the colonies are dark green to brown or black covered with a feltlike aerial mycelium with individual strains showing variation in rate of growth and gross character of the colony (Fig 99) Microscopically *H pedrosoi* varies greatly in its method of conidial formation Three different methods of sporulation are recognized conidia in branching chain formation from conidiophores of varying length (*Hormodendrum* type) (Fig 99) conidia surrounding the swollen knotted club shaped terminal ends of hyphae (*Acrotheca* type) (Fig 99) and conidia produced endogenously from flask shaped conidiophores with a terminal cup (*Phialophora* type) Different strains show a predominance of one type of sporulation

H compactum grows slowly on Sabouraud's glucose agar producing a heaped brittle colony olive black in color with tufts of coarse aerial mycelium (Fig 100) Microscopically chains of spherical to subspherical conidia in compact sporulating heads distinguish this species from *H pedrosoi* (Fig 100) The *Phialophora* type of sporulation is also found in cultures of *H compactum*

Phialophora verrucosa on Sabouraud's glucose agar produces a dark brown to black colony with short feltlike olivaceous gray aerial mycelium (Fig 101) This fungus reproduces by a single type of sporulation Microscopically flask shaped conidiophores borne terminally or laterally from the hyphae produce conidia singly at the tip within a cuplike structure (Fig 101) These conidia successively produced at the tip of the conidiophore usually remain in clusters in undisturbed preparation



Fig 102 *Hormodendrum pedrosoi* section of skin showing pigmented round fruiting bodies of fungus in giant cell × 49

Distribution

Chromoblastomycosis is world wide in distribution with cases having been reported from every continent with the exception of Asia Although the disease is found in temperate climates the vast majority of cases have occurred in the tropics So far as is known it is only a human disease Monkeys dogs rabbits guinea pigs rats and mice have been inoculated by various routes with only occasional reports of experimental infection

Pathogenesis

Chromoblastomycosis is a chronic slowly developing (months to years) unilateral granulomatous infection of the skin which develops following introduction of the fungus by trauma In the majority of cases the lesions appear on the lower extremities but occasional cases have been reported where lesions appeared on the hand the arm the face the neck and the buttock In the typical case the lesions are unilateral and are first [illegible] warty growths which extend slowly a [illegible] lesions along the lymphatics Over a period of months the lesions enlarge to become [illegible] ing papillomatous verrucous elevated [illegible] with a cauliflowerlike appearance Elephantiasis of the affected member usually [illegible] marked fibrosis and lymph stasis [illegible] are unknown and regional a[illegible] from secondary [illegible] The [illegible] nature of the infection [illegible] it is seen that lesions first

ing the typical appearance described above must be differentiated from other mycotic diseases tuberculosis verrucosa cutis late nodular syphiloderm epithelioma leishmaniasis, yaws mossy foot etc

Histologically the lesions are granulomatous with pseudotubercle formation The epidermis shows marked hyperplasia extensive hyperkeratosis parakeratosis and acanthosis with elongated distorted rete pegs The corium contains pseudotubercles made up of epithelioid cells and giant cells of the Langhan s type in the center of which are polymorphonuclear leukocytes Dark brown fungus bodies are found among the polymorphonuclear cells or in the giant cells Surrounding these tuberculoid granuloma are lymphocytes plasma cells eosinophils and a few polymorphonuclear cells

Immunity

Positive complement fixation tests have been reported by Conant and Martin (1937) Since this test is difficult to perform it is not a practical diagnostic aid Diagnosis is established more readily by finding the organism in clinical materials and by culture

Diagnosis

Epidermal debris from the cauliflowerlike nodules should be examined in 10 per cent potassium hydroxide preparations for the presence of the dark brown septate bodies 6 to 12 μ in diameter characteristic for chromoblastomycosis Sections from biopsies also should be examined for these bodies and the typical histopathologic reaction (Fig 102) Both these materials should be cultured on Sabouraud s glucose agar at room temperature Since all of the etiologic agents mentioned above produce identical bodies in tissue the causative agent of a given case can be established only by the isolation and identification of the fungus

Treatment

Small localized lesions may be removed successfully by surgical excision cauterization or electrocoagulation Iodide by mouth or intravenously with or without x ray treatment locally have been used with variable results One extensive case was treated by iontophoresis with copper sulfate with good results (Martin, Baker and Conant 1936)

Epidemiology

Chromoblastomycosis is a disease of the skin of the exposed parts of the body It is most frequent in the tropics among barefooted agricultural laborers and others with close contact with the soil The fungi are saprophytes in nature and enter the skin by trauma The disease is not transmitted from man to man is more prevalent during adult life (30 to 50 years of age), is rarely reported in females and shows no racial immunity

DERMATOPHYTES

The dermatophytes are a closely related group of fungi which cause specific infections of man and animals by invading only the superficial keratinized areas of the body such as the skin, the hair and the nails They do not cause systemic infections and rarely invade the subcutaneous tissues In their parasitic habitat they show a very reduced rudimentary morphology appearing only as mycelial fragments in skin and nails or as mycelial fragments and arthrospores arranged inside or outside the hair However in culture on Sabouraud s glucose agar at room temperature they develop filamentous colonies which reproduce by a variety of spore forms characteristic of the group Three genera are now recognized *Microsporum Trichophyton* and *Epidermophyton*

History

Schonlein 1839 reported the first etiologic agent of disease in man when he described a fungus as the cause of favus Remak 1845, named this fungus *Achorion schoenleinii* Within a few years, other fungi were reported as the etiologic agents of disease in man Gruby 1843 described *Microsporum audouini* and Malmsten 1845 described *Trichophyton tonsurans* as etiologic agents of ringworm of the scalp Later Sabouraud 1907 described *Epidermophyton inguinale* from eczema marginatum while Castellani 1910 reported *Endodermophyton concentricum* from tinea imbricata or Tokelau Complete descriptions of all types of ringworm infection of the hair the skin and the nails as well as the fungi which caused such lesions were published by Sabouraud 1910 who listed 45 species of dermatophytes More than 100 species of *Trichophyton* alone are now

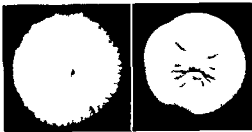


FIG 103 (*Left*) *Trichophyton* (*gypseum*) *mentagrophytes* 14 days on Sabouraud's glucose agar at room temperature (*Right*) *Trichophyton* (*interdigitale*) *mentagrophytes* 14 days on Sabouraud's glucose agar at room temperature



FIG 104 Microscopic morphology of *Trichophyton mentagrophytes* (*Left*) Conidiophores producing clusters of microconidia (en grappe) × 368 (*center*) Microconidia borne laterally on hypha (en thyrses) × 368 (*right*) Macroconidium (fuseau) × 368

listed in the literature These species were separated not only on the basis of differences in the appearance of lesions from which they were isolated but also on differences observed in the gross appearance of colonies on Sabouraud's standard medium However critical studies of these dermatophytes have reduced 2 genera and several species to synonymy with previously described forms Thus the genera *Achorion* and *Endodermophyton* have been discarded and their species have been placed in the genus *Trichophyton* This genus now contains only 12 species while *Microsporum* contains only 3 species and *Epidermophyton* a single species (Conant et al 1954)

CULTIVATION

The dermatophytes may be grown on a variety of simple media but they are usually cultivated on Sabouraud's glucose agar at room temperature because the somewhat typical appearance of the colonies and the microscopic morphology developed on this medium have been used for generic and specific identification Recent studies of the exact nutritional requirements for some of the dermatophytes have resulted in a more stable taxonomy of these fungi by allowing better colony formation more consistent spore production and less variable macroscopic appearances

Genus *Trichophyton* On Sabouraud's glucose agar at room temperature the colonies are granular to powdery cottony to velvety heaped wrinkled and folded with a velvety surface or heaped wrinkled and folded with a smooth and waxy surface Pigmentation of the colonies varies from delicate pink to red purple violet brown yellow and light buff

Some of the species in this genus (*T verrucosum* *T schoenleini* *T tonsurans* *T megnini* *T equinum*) can be distinguished from each other and from all other dermatophytes by their cultural requirements Physiologic tests to determine these requirements correlated with gross colony formation on Sabouraud's glucose agar has allowed easier and more nearly accurate identification of these so called difficult to identify dermatophytes (Ajello 1957 Georg 1957)

Microscopically microconidia are the prominent spore forms These are subspherical pyriform or clavate (1 5 to 2 x 2 to 5 μ) distributed on the sides of the hyphae (en thyrses) or produced on conidiophores in clusters (en grappe) Macroconidia are characteristic but rare and appear as long thin walled multiseptate clavate spores (4 to 6 μ in width x 10 to 50 μ in length) Raquette mycelium nodular bodies coiled hyphae and chlamydospores are also found in some species

TRICHOPHYTON (GYPSEUM) MENTAGROPHYTES (Robin) Blanchard 1896 Primary cultures may be granular to powdery and light buff to tan in color Occasional strains which produce a pink to red pigment in Sabouraud's glucose agar can de differentiated from *T rubrum* by cultivation on corn meal glucose agar in which red pigment is not produced (Bocobo and Benham 1949) Over growth of fluffy cottony pure white mycelium on transfer produce the interdigitale type

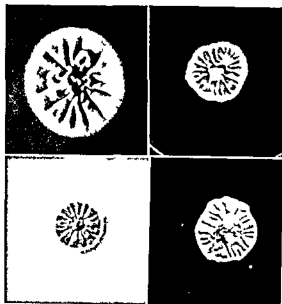


FIG 105 (*Top left*) *Trichophyton tonsurans* 35 days on Sabouraud's glucose agar at room temperature (*top right*) *Trichophyton rubrum* 12 days on Sabouraud's glucose agar at room temperature (*bottom left*) *Trichophyton violaceum* 19 days on Sabouraud's glucose agar at room temperature (*bottom right*) *Trichophyton concentricum* 12 days on Sabouraud's glucose agar at room temperature



FIG 106 (*Left*) *Trichophyton schoenleini* 21 days on Sabouraud's glucose agar at room temperature (*Right*) Favic chandeliers produced in cultures of *T schoenleini* × 183

of colony (Fig 103) Sporulation on Wort agar as well as on Sabouraud's glucose agar aids in the identification of this species Microscopically numerous subspherical microconidia tightly coiled hyphae chlamydospores raquette hyphae and nodular bodies but few macroconidia are seen (Fig 104)

T (PURPUREUM) RUBRUM (Castellani) Sabouraud 1911 Primary cultures are cottony and pure white but later develop a velvety surface with a rose purple or reddish pigment on the back of the colony (Fig 105) Pigmentation may spread into the agar and into the surface mycelium This pigmentation is more constant when cultures are grown on corn meal glucose agar and macroconidia are more abundant when cultures are grown on blood agar base Difco (Benham 1948) Microscopically numerous clavate microconidia borne on the sides of the hyphae chlamydospores and raquette hyphae but few macroconidia are seen

T (CRATERIFORME) TONSURANS Malmsten 1845 The colony is slow growing with compact whitish cream velvety surface that becomes folded with deep crateriform depressions of yellowish color (Fig 105) Stimulation of this species by the addition of thiamine to the medium distinguishes it from *T mentagrophytes* and *T rubrum* above Macroconidia are more abundant in cultures on Wort agar Microscopically, numerous clavate microconidia borne on the sides of the hyphae numerous chlamydospores hyphal swellings and raquette hyphae but rare macroconidia are seen

T (ACHORION) SCHOENLEINI (Lebert) Langeron and Milochevitch 1930 The colony is slow growing heaped compact waxy and smooth with many irregular folds yellowish white to light brown in color (Fig 106) This species is autotrophic for vitamins and is distinguished from *T verrucosum* (below) on this basis On transfer the smooth waxy appearance changes to a velvety white Microscopically only chlamydospores hyphal swellings and the so-called favic chandeliers are seen (Fig 106)

T (ACHORION) VIOLACEUM Sabouraud 1902 The colony is slow growing heaped compact smooth waxy, with irregular folds and a deep violet pigmentation (Fig 105) Better growth occurs when thiamine is added to Sabouraud's glucose agar or when this species is grown on blood agar base medium plus thiamine Microscopically only chlamydo

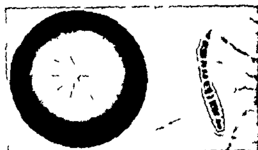


Fig 107 *Microsporum audouini* (*Left*) Twenty one days on Sabouraud's glucose agar at room temperature (*Right*) Macroconidium (fuseau) elongate imperfectly formed macroconidia are found in this species × 351



Fig 108 *Microsporum canis* (*Left*) Fourteen days on Sabouraud's glucose agar (*Right*) Well developed and numerous macroconidia are found in this species × 350

spores and hyphal swellings with no characteristic spore forms are seen

T (ENDODERMOPHYTON) CONCENTRICUM Blanchard (1896) The colony is slow growing heaped deeply furrowed smooth and brownish in the center (Fig 105) On transfer the surface becomes velvety Fifty per cent of the strains studied are found to be autotrophic for the vitamins while 50 per cent required thiamine for good growth Microscopically only chlamydospores hyphal swellings and the so-called favic chandeliers are seen

T (MICROSPORUM) FERRUGINEUM (Ota) Langeron and Milochevitch 1930 The colony is slow growing glabrous smooth and orange in color This species is autotrophic for the vitamins Microscopically only chlamydospores and hyphal swellings are seen

T VERRUCOSUM Georg 1950 The colony is slow growing convex disklike moist glabrous and dull yellow in color This species requires thiamine or thiamine and inositol for good growth Microscopically only chlamydospores and hyphal swellings are seen

Genus *Microsporum* On Sabouraud's glucose agar at room temperature the colonies are slow growing matted and furrowed or fast growing cottony or powdery and tan to cinnamon brown in color The pigmentation in the agar may be reddish brown to orange Microscopically the macroconidia are numerous and characteristic They are large (8 to 15 μ in width × 40 to 150 μ in length) spindle shaped multicelled rough thick walled spores The microconidia (2 5 to 4 × 3 to 6 μ) scarce in primary cultures are borne singly along the hyphae or from short stalks from the hyphae

M AUDOUINI Gruby 1843 The colony is slow growing matted and velvety tan to brownish in color with yellowish or orange pigmentation in the agar (Fig 107) Yeast extracts glucose and a paramine added to medium provokes vegetative growth This species does not produce spores readily which would allow easy identification Lack of growth on a polished (unfortified) rice distinguishes it from other species of dermatophytes Microscopically the macroconidia are rare and when found in occasional isolates are bizarre in shape The microconidia are clavate (2 5 to 4 × 3 to 6μ) borne on the hyphae or from short stalks on the hyphae Pectinate hyphae raquette mycelium chlamydospores and nodular bodies are also seen

M CANIS Bodin 1902 The colony is fast growing with abundant cottony aerial buff tan mycelium and yellowish to orange pigmentation in the agar (Fig 108) Microscopically numerous characteristic macroconidia are produced They are large (8 to 15 × 40 to 150 μ) multicelled spindle shaped thick walled spores (Fig 108) Raquette mycelium chlamydospores and nodular bodies are also seen

M GYPSEUM (Bodin) Guiart and Grigorakis 1928 The colony is fast growing with white cottony aerial mycelium which becomes matted and powdery and cinnamon brown in color (Fig 109) Microscopically numerous macroconidia are produced They are elongate and ellipsoid (8 to 12 × 30 to 50 μ) multicelled rounded to tapering at the ends with rough thin walls (Fig 109)

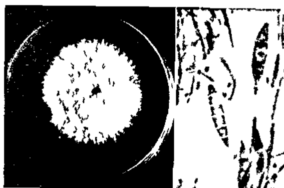


FIG 109 *Microsporum gypseum* (*Left*) Seven days on Sabouraud's glucose agar at room temperature (*right*) Macroconidia × 330





FIG 110 *Epidermophyton floccosum* (*Top*) Twelve days on Sabouraud's glucose agar at room temperature (*Bottom*) Typically clustered macroconidia × 5?0

Raquette mycelium, chlamydospores and nodular bodies are also seen

Genus Epidermophyton On Sabouraud's glucose agar at room temperature the colonies are velvety to powdery and greenish yellow in color Microscopically only oval to broadly clavate macroconidia are produced This genus contains one species

E FLOCCOSUM *(Harz) Langeron and Milochevitch 1930* The colony develops with a central cottony white aerial mycelium which becomes powdery and greenish yellow in color (Fig 110) Microscopically, the oval, broadly clavate 2 to 6 celled smooth thin walled macroconidia (7 to 12 x 20 to 40 μ) are characteristic for this fungus They are produced directly from the hyphae or in typical clusters (Fig 110) No microconidia are to be found Older cultures produce many chlamydospores and raquette cells

DISTRIBUTION

The dermatophytes have a worldwide distribution However some species are found constantly in certain geographic area and rarely in others

T (Achorion) schoenleini is found in the countries bordering the Mediterranean in the Balkans and scattered throughout Europe and the Far East Cases of infection in this country usually are found in families of recent immigration *T (Achorion) violaceum* also is found in the Balkans Russia and southern European countries with scattered cases reported throughout Europe In the United States, cases of infection by this fungus are sporadic and usually found in foreign families *T (Microsporum) ferrugineum* is common in Manchuria and Japan and rarely found elsewhere *T (Endodermophyton) concentricum* seems to have a tropical distribution it has been reported from the Pacific South America and occasionally from Central America As yet no cases have been reported from the temperate zone *T (purpureum) rubrum* is said to be more prevalent in subtropical areas (Mexico Central America, West Indies parts of South America) In the United States more cases are reported from the southern part of the country

M audouini is endemic in Europe (France Spain Italy Germany Austria and in the Balkans) Until World War I the occurrence of this fungus was sporadic in England and the United States During World War II however, epidemics caused by *M audouini* in

the United States and Canada have caused great concern

Many of the dermatophytes cause infections in animals and their range of pathogenicity is extensive. The cat, dog, horse, calf, cow, sheep, squirrel, monkey, rat, etc., have been found to have spontaneous infections with one or another of the dermatophytes and laboratory animals such as the guinea pig and the rabbit, as well as the cat and the dog, may be infected experimentally. Positive blood cultures may be obtained occasionally from guinea pigs with experimentally induced skin lesions, but these animals do not show internal lesions. Intravenous injection of guinea pigs with spore suspensions does not result in infection of the internal organs, but specific cutaneous lesions will be formed if the skin of such animals is traumatized. This extreme specialization for and invasion of a particular tissue is not duplicated by any of the other pathogenic fungi. Some fungi, such as *M audouini*, are considered to be human species in that they are rarely found on animals, and animals rarely can be infected experimentally.

Pathogenesis

The dermatophytes cause superficial infections (dermatomycoses) of the keratinized areas of the body, i.e., skin, hair and nails. They do not invade the deeper tissues or internal organs of man and do not cause systemic infections in experimentally inoculated animals.

The most prevalent infection is that referred to as tinea pedis (athlete's foot, dermatophytosis, etc.), in which the toe webs are invaded by species of *Trichophyton* or *E floccosum*, resulting in acute, subacute or chronic infections. In most instances the infection becomes noticeable as a pruritic vesiculated area between the toes with occasional spread to the rest of the foot. Rupture of the vesicles and discharge of a thin serous fluid causes maceration and peeling of the tissue. This may be accompanied by the appearance of fissures or cracks. Unless secondary bacterial infection takes place, the lesion usually persists for long periods of time as a macerated area between the toes. However, superimposed bacterial invasion may result in an acute inflammatory reaction with lymphangitis or lymphadenitis. Occasionally certain species of *Trichophyton* cause marked inflammatory reactions and the fungus or its products sensitize the skin. In such cases vesicular lesions indistinguishable from primary infections may appear elsewhere on the body, particularly on the palms of the hands. These lesions are considered to be allergic manifestations or dermatophytids if fungi are not found in them, a primary focus of infection is found elsewhere on the body, and the trichophytin test is positive.

Infection of the nails (tinea unguium) may accompany lesions between the toes or on the feet. Usually only 2 or 3 nails are affected and these become discolored, brittle, opaque, lusterless, thickened and friable. Paronychia is not common.

Infection of the glabrous skin of the body (tinea glabrosa) occurs more commonly in children as a result of contact with infected animals or by autoinoculation with hairs from an infected scalp. Adults may become infected by handling animals or infected children or from lesions on the nails and the feet. Although a variety of lesions on the glabrous skin may be caused by dermatophytes, the typical annular ringworm lesion is one with a healing scaly center and active erythematous vesiculopustular border.

Ringworm of the scalp (tinea capitis) occurs in childhood and in most instances, if not cured during this period, heals spontaneously at puberty. However, a few of the dermatophytes cause lesions which tend to hold over into adult life (*T schoenleini*, *T violaceum*, *T tonsurans*). Infection by *M audouini* is acquired by contact with other infected children and usually occurs in epidemics. However, infection by *M* (*lanosum*) *canis* is acquired by contact with infected animals (cats and dogs) and is usually sporadic. The appearance of the lesions depends on the infecting fungus, whether a *Microsporum* or a *Trichophyton*.

In microsporosis the hair is broken off a short distance from the surface of the scalp, leaving grayish areas composed of hair stubs surrounded by a sheath of spores. Infection by *M canis* or *M gypseum* may also cause an inflammatory reaction resulting in a boggy tumorlike mass or kerion which resembles a pyoderma. In trichophytosis, species of



FIG 111 Potassium hydroxide preparation of skin and hair (*Top left*) *Trichophyton mentagrophytes* in skin × 415 (*top right*) *Trichophyton* (endothrix) hair × 170 (*bottom left*) *Microsporum* hair × 170 (*bottom right*) *Trichophyton* (*Achorion*) hair × 170

Trichophyton which invade the hair shaft (*endothrix*) cause small scattered scaly lesions with a thinning of the hair where they are broken off at the surface of the scalp leaving follicles with a black center (black dot ringworm) Another endothrix species *T schoenleini* causes a characteristic infection of the scalp (favus) characterized by cuplike structures (scutula) formed by the infected hair follicles Ectothrix species *T* (*gypseum*) *mentagrophytes* may produce acute inflammatory reactions resulting in kerion formation

Infection of the bearded region of man (tinea barbae) may be caused by various species of *Trichophyton* and resemble closely infections due to pyogenic organisms

Since the dermatophytes invade the horny layer of the epidermis and can live and multiply only in this dead skin there are few histopathologic changes that are not those of a response to any inflammatory reaction These fungi cause erythema and edema with inflammation, resulting in scaling of the stratum corneum and vesiculation Microscopically there is a marked hyperkeratosis parakeratosis, acanthosis, and dilatation of the vessels of the papillary layer with plasma and cellular infiltration resulting in interstitial edema

IMMUNITY

Children are susceptible to infection of the scalp and the body by dermatophytes of human or animal origin but are resistant to infection of the feet Adults on the other hand are susceptible to infection of the feet, the nails and glabrous skin but are relatively resistant to infection of the scalp (Kligman and Ginsberg 1950)

Greenbaum, 1924 failed to demonstrate circulating antibodies by means of the complement fixation test Marcussen 1937 by the Prausnitz Kustner technic demonstrated circulating antibodies of the urticarial type in individuals with allergic manifestations who gave an immediate wheal to intracutaneous injections of trichophytin

This immediate allergic type reaction has been found to occur not only in the atopic individual but also in individuals infected by *T rubrum* or with a history of recurrent lymphangitis (Jillson and Huppert, 1949) However immunity in humans is usually demonstrated by the cutaneous sensitivity which is established during infection and can be demonstrated by an intracutaneous injection of trichophytin The reaction is of the delayed type (24 to 48 hours) and frequently lasts 7 days A positive trichophytin test depends to some extent on the type of invading fungus and whether or not an inflammatory reaction is induced The trichophytin used to elicit the skin test in sensitive individuals contains both group specific and species specific antigens The trichophytin test may indicate either present or past infection by any one of the dermatophytes

Immunity in infected animals can be demonstrated not only by a cutaneous reaction

to trichophytin but also by an acute accelerated type of lesion produced on reinoculation (De Lamater and Benham 1938 De Lamater 1941) Circulating antibodies have not been demonstrated in infected animals and only rarely in immunized animals Keeney and Eriksen (1949) demonstrated specific precipitins to protein fractions of *T rubrum* and *T mentagrophytes* in the sera of rabbits immunized by intravenous injections of live spores Wharton et al (1950) showed rabbits to be immune to infection and to produce precipitins to a saline extract of dried alcohol treated cultures when immunized with an oil emulsion of killed cultures and killed tubercle bacilli

Diagnosis

Diagnosis is made best by demonstrating the fungus in the hair the skin or the nails and by culture The Wood s light is an invaluable aid in locating and determining the extent of infection on the scalp Hairs infected with *M audouini* and *M canis* fluoresce when this light is held close to the scalp in a semidark room Hair epilated from lesions on the scalp skin scraped from the erythematous border of lesions on the skin or obtained from the roofs of vesicles and scrapings obtained from the discolored friable areas of infected nails are examined in 10 per cent KOH Dermatophytes in the skin or the nails appear as branching fragments of hyphae (Fig 111) the genus and the species of the invading fungus can be determined only by culture In the hair the appearance of these fungi in KOH preparations allows to some extent a generic determination Species of *Microsporum* form dense spore sheaths around the hair stub with the spores crowded into a mosaic pattern (Fig 111) Species of *Trichophyton* form parallel rows of small or large spores outside the hair shaft (ectrothrix microides ectothrix megaspores) or inside the the hair shaft (endothrix) (Fig 111) Although the appearance of the infected hair may allow identification of the genus of dermatophyte the species can be identified only by culture

Cultures are made by inoculating Sabouraud s glucose agar slants with 2 or 3 fragments of infected material The addition of penicillin streptomycin and cycloheximide (actidione) to the medium will greatly increase the percentage of positive cultures by eliminating bacterial and faster growing fungus contaminants (Georg et al 1954) All cultures must be maintained for at least 3 weeks at room temperature before being discarded as negative

Treatment

The treatment of scalp infections must be directed toward the epilation of all infected hairs The hair should be clipped as closely as possible and with the aid of a Wood s light the infected area should be outlined with gentian violet or other suitable dye Manual epilation of hairs within the infected areas should be attempted 3 or 4 times weekly The head should be shampooed daily followed by an application of Salicylanilide ointment copper undecylenate ointment or other topical remedies A clean cotton stocking cap (made from the top of a woman s stocking) should be worn at all times and changed daily Such caps must be boiled for at least 10 minutes before washing Progress of treatment should be followed by weekly examinations under a Wood s light after thorough shampooing of the scalp Infections caused by *M canis* should respond in 2 to 3 months Infections caused by *M audouini* may respond in 3 to 6 months but usually persist until puberty at which time the infection heals spontaneously Epilation by x rays followed by the above routine is the best method of treatment for infections caused by *M audouini* and *T tonsurans*

Infections of the glabrous skin usually respond easily (2 to 3 weeks) to treatment with tincture of iodine (1 to 3 5%) ammoniated mercury (5%) or a sulfur salicylic ointment (3% each) Infections due to *T* (*purpureum*) *rubrum* or *T schoenleini* demand vigorous treatment over a longer period of time

Acute infections of the feet should be treated conservatively until all signs of inflammation have disappeared Continuous compressing with warm boric acid solution 1 4 000 potassium permanganate solution or physiologic saline should be followed by manual debridement of macerated tissue all vesicles and bullae should be opened When the acute process has subsided a sodium propionate ointment or an undecylenic acid

zinc undecylenate ointment may be applied at night During the day a dusting powder may be used (sodium propionate 20% in talc 80% zinc undecylenate 20% undecylenic acid 2% in talc 78% Every effort should be made to avoid overtreatment in fungus infections of the feet

Epidemiology

The dermatophytes include species which primarily infect animals and only incidentally infect humans (animal or zoophilic species) and species which infect humans only (human or anthropophilic species) The animal species e g *M canis* from dogs and cats or *T verrucosum* from cattle cause sporadic cases of ringworm of the scalp or glabrous skin of children or the glabrous skin of adults Usually such infections can be traced to infected cats or dogs The human species *M audouini* causes epidemics of ringworm of the scalp of children by person to person spread of hairs infected with this fungus Such hairs are easily dislodged from the scalp and may be picked up from the backs of theater seats from the clippers in barber shops or by direct contact at play Since the duration of treatment is long (3 to 6 months) and the incidence of family infections and preschool infections is high no attempt is made to keep infected children from attending school Epidemics caused by *M audouini* recently have reached such proportions that the disease can be handled best only by Public Health facilities

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habitat The greater variety are closely related to the more characteristic freely communicable human pathogenic bacteria By contrast *animal pathogens that infect man* aberrantly and are not usually communicated from man to man like *Pasteurella Brucella* and *Malleomyces* do not have relatives in the indigenous biota These circumstances can hardly be accidental They suggest an evolutionary adaptation of both participants from the lesser security of pathogenicity toward the greater one of amphibiosis

In this context the word infection may need redefinition All that it implies strictly is an interaction of a host organism with a smaller self replicating agent such that both are modified in the process Beyond the view now gaining currency in medicine that infection can occur without disease as in tuberculosis poliomyelitis and notably with the Echo and adenoviruses is the suggestion that disease is only one of several possible consequences of infection Thornton (1952) considered the symbiotic relationship of *Rhizobium* with legumes as infection and Ravin (1955) suggested that fertilization can be excluded from the scope of the term only arbitrarily

RANGE OF THE INDIGENOUS BIOTA

The most characteristic members of the indigenous biota are bacteria Protozoa fungi pleuropneumonialike forms and even viruses may be considered as indigenous but each of these poses distinctive problems which are not dealt with here for lack of space

THE INDIGENOUS BACTERIA

The following bacterial groups include cultivable forms that appear to be indigenous to man Only the more clearly defined species that are either widespread or conspicuous because of concentration or distinctive properties or both are noted The listing includes some true saprophytes but excludes recognized pathogens

Micrococcus Aerobic staphylococci typically but not invariably coagulase and mannite negative nonhemolytic and white (*M epidermidis*—*M albus*) are characteristic on skin and are frequently found probably in low concentrations on mucous surfaces Hite et al (1947) found them in the vagina in 34 per cent of 61 cases only aciduric rods and diphtheroids were observed more commonly These forms merge with the pathogenic *M aureus* (See Jordan et al [1956])

Obligately anaerobic micrococci continue to be described among the indigenous biota especially of the mouth the upper respiratory tract and the vagina but their status and classification are doubtful

Among 99 strains of anaerobic cocci mainly from the vagina and the respiratory tract Hare et al (1952) found features suggestive of *Micrococcus* (or *Staphylococcus*) but none could be identified unequivocally with forms described by earlier workers A group of 36 strains of strictly anaerobic micrococci studied by Foubert and Douglas (1948) were catalase positive Prevot and Thouvenot (1952) have noted that catalase activity when found among strains (of diphtheroids) which were strictly anaerobic when isolated *was associated with adaptation to growth in air*

Streptococcus Aerobic streptococci lacking the group A polysaccharides are highly characteristic of mucous surfaces especially in the mouth the pharynx and the lower intestine

Among them the enterococci including *S faecalis* and other species (see Shattock 1955) can be distinguished by the presence of the group D carbohydrate Nearly all of these also grow at 10 and at 45 at pH 9 6 in 6 5 per cent NaCl and in 40 per cent bile They usually withstand 60 for 30 minutes and are generally resistant to antibiotics Other characteristics including hemolytic activity are variable Indirect evidence suggests that enterococci may be as numerous in feces as *E coli* Indigenous streptococci lacking either D or A substances have been grouped as viridans streptococci They are typically greening on blood but many are anhemolytic and some show β hemolysis This group is found especially in the mouth the throat and the vagina and is often accepted as predominant in the first two areas but they appear to be outnumbered by strict anaerobes *S salivarius* (or *S hominis*) is probably the most clearly defined member of this group Identified principally by the production of large mucoid colonies on 5 per cent sucrose (or raffinose) agar—dependent on production of a soluble levan—it is also usually inactive on blood grows at 45 but not at 10 ferments inulin salicin and esculin and acidifies

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Bacteria Indigenous to Man

From the moment of birth and throughout life man is exposed to an environment laden with micro-organisms. The resultant of our contact with them is inevitable interaction with effects that may take a seemingly continuous range of forms depending on the distinctive reactivity of the micro-organisms. True saprophytes (micro-organisms indigenous, e.g., to soil) with a few exceptions, fail to maintain themselves on human tissues; the interaction entails a presumably active although grossly inappreciable host component and leads to destruction of the micro-organism. If the microbe is parasitic and highly adapted to an animal species other than man but is nevertheless able to proliferate in human tissues, the resultant of appropriate contact with man is likely to include a manifest host response recognizable as overt disease. Between these extremes are microbes that are highly adapted to growth in or upon human tissues. The indigenous microbes fall into this group, but so do many agents recognized as "pathogens." No sharp distinction between the two classes can be drawn. "Pathogens" may achieve equilibrium with the host either from the start (as with "healthy" carriers of pneumococci or hemolytic streptococci) or after an initial upset, as with a wide and familiar range of infective diseases. With the indigenous microbes equilibrium is the rule. Perhaps they are the more highly adapted, so that disease, if it occurs at all, results only from a disturbance of the equilibrium. There is a continuous spectrum from the imminent or potential pathogenicity known as latency through apparently permanent neutrality (the resultant of the interaction being undetectable) to symbiosis. The word "commensalism" is often applied to this intermediate range, but like other terms that grew out of an early and necessarily rigid interpretation of the germ theory, in which the focus was sharply on disease and a simple cause-and-effect relationship, "commensalism" implies a passivity, and by corollary an inaccessibility to research, that now seem to be unsophisticated. A better term, borrowed from vertebrate zoology, may be "amphibiosis" — including both symbiosis and antibiosis (or pathogenicity) or anything between the two.

The indigenous bacterial biota of man may thus be characterized as occupying a band of a continuous spectrum between saprophytes and pathogens, typically neither but merging with both. In the middle of the band the biota consists of obligate parasites which in pure culture lack obvious or easily demonstrable pathogenicity. Some of them are distinctive species known to occur only in this

tion (Johns 1951) It fails to attack sugars but ferments lactate actively under anaerobic conditions with the production of propionic and acetic acids and large amounts of CO and H accompanied by a rise in pH Pyruvate malate fumarate oxaloacetate succinate and tartrate are also fermented The organism is apparently nonpathogenic it is thought to play a significant role in the ovine rumen in the formation of propionic acid from lactate Its regular presence in saliva in high concentration can hardly be insignificant but remains unexplained

Lactobacillus The lactobacilli are associated with important activities in the mouth the lower intestine and the vagina The most numerous and frequent varieties found in the mouth (other than *L acidophilus*) and probably tho e of the other two areas as well are now known to be saprophytes

The classification of oral lactobacilli has been clarified by the studies of Rogosa et al (1953) and others who have hown that the most commonly found forms are indistinguishable from those recovered from natural fermentations Although these studies have not been extended to the intestinal lactobacilli the earlier work of Harrison and Opal (1944) which indicated that the fecal lactobacilli re emble both in concentration and in type distribution those found in the mouth of the corresponding individual suggests that the same principles may apply The aerobic lactobacilli of the vagina have not been identified in line with the current classification Both homofermentative and heterofermentative lactobacilli have been recovered from the mouth the latter being distinguished in practice by the production of gas from glucose Only the commonest pecies need be listed here The descriptions are from Davis et al (1955) Nearly half of the oral strains are *L casei* a homofermentative form characterized usually by a smooth colony curved bacilli often in curling chains and growth at 15 but usually not at 45 Mannite salicin and mannose are fermented and hippurate and esculin hydrolyzed no NH_3 is formed The homofermentative parasitic *L acidophilus* accounts for 10 per cent or less of oral strains Its colony is small and rough its cells variable straight usually rather long Growth occurs at 45 but not at 15 NH_3 is not produced Typical strains ferment salicin lactose melibiose and mannose and hydrolyze esculin Among heterofermentative species *L fermenti* is the most common representing some 40 per cent of oral isolations The colony is usually smooth and relatively large the cells straight and short This species grows at 45 but usually not at 15 It forms NH_3 usually hydrolyzes esculin and hippurate and tolerates 10 per cent bile *L brevis* another gas forming species comprises some 5 per cent of oral isolations has a rough or intermediate colony and is distinguished otherwise by failing to form NH_3 or to tolerate 10 per cent bile The homofermentative pecies as would be expected are more active acid producers than the gas formers

ANAEROBIC LACTOBACILLI Anaerobic lactobacilli including both strict anaerobes and forms that become adapted to aerobic growth are found principally in the intestinal contents of nursing infants but have al o been recovered from the mouth and the vagina These organisms some of which show rudimentary branching (bifid forms) appear to be a group of obligate parasites that intergrade with *L acidophilus* on the one hand and with *Actinomyces israeli* on the other but a further subdivision is suggested by the finding (Norris et al 1950) that some of the unbranched anaerobic lactobacilli produce large amounts of gas (CO)

Specific designations in this group are ambiguous the terms *L bifidus* and *L parabifidus* having been u ed interchangeably Pine and Howell (1956) compared 12 strains of anaerobic or microaerophilic actinomycetes with 4 strains of *L bifidus* the latter presumably all branched non gas forming strict anaerobes The two groups were remarkably similar to their biochemical characters but two distinctions were noted (1) the actinomycetes reduced NO_3 the lactobacilli did not and (2) the lactobacilli produced larger quantities of acetic acid from gluco e apparently by utilizing a separate metabolic pathway

Corynebacterium The aerobic corynebacteria of mucous membranes are conventionally classified principally by fermentation of glucose and sucrose

The pathogenic *C diphtheriae* ferments glucose but not sucrose *C hofmannii* (*C pseudodiphtheriticum*) originally found in the throat ferments neither *C xerose* originally isolated from the conjunctiva ferments both

and clots litmus milk Williams (1956) found that 60 of 103 levan producing oral pharyngeal streptococci reacted with group K antiserum whereas none of 13 old group K strains including 3 from Hare s collection formed levan Williams gives cogent reasons including priority of the specific epithet (proposed by Hlava in 1902) for renaming the levan producing streptococci *S hominis* independently of the K antigen The remaining indigenous aerobic streptococci are a heterogeneous group that has not yet been classified satisfactorily The name *S mitis* is applied loosely to those forms, commonest in mouth and throat that fail to produce levan on sucrose agar and do not ferment inulin Most strains are greening on blood Other specific designations among indigenous streptococci are of doubtful utility The Lancefield polysaccharides C E, F G H and O as well as K not necessarily associated with β hemolysis or other properties have been found distributed among some of the apparently nonpathogenic streptococci of mouth and throat Of the other characters including dextran formation from sucrose (which is not associated with mucoid colonies) action on red blood cells fermentation reactions or other metabolic or nutritional phenomena and resistance to deleterious agencies none has been accepted as a taxonomic index

Strictly anaerobic streptococci are doubtlessly indigenous widespread and probably numerous on mucous membranes but our knowledge of them is characteristically inadequate

Prevot listed 9 species with additional varieties in 1948 classified in two groups which either (1) produced or (2) failed to produce gas and a fetid odor Among the second group a single species *S evolutus* was said to become adapted to aerobic growth the others were strict anaerobes This grouping is in line with the findings of Hare et al (1952) who reported isolating 45 strains mainly from the vagina of a member of the first group *S putridus* This organism ferments glucose fructose and maltose with the production of large volumes of gas principally CO the gas appearing only in the presence of S containing compounds Other metabolic products according to Prevot are NH_3 H S and formic butyric and acetic acids *S putridus* is a rather large coccus with a tendency to pleomorphism showing clubbed and bacillary forms together with variable cocci in individual chains The name *S micros* may be used provisionally to cover Prevot s second group This organism or group first described by Lewkowicz in 1901, may be characterized as a variably small coccus (0 3 07 μ diam) producing no gas or odor It fails to liquefy gelatin, does not form indol but produces NH_3 carbohydrates are attacked with the production of lactic propionic and formic acids and usually acetylmethyl carbinol

Neisseria The indigenous aerobic oxidase positive gram negative cocci, most characteristic of the pharynx but also found apparently sparsely, on other mucous surfaces including urethra and vagina, are grouped into 3 species

N catarrhalis is the unpigmented form that fails to produce acid from any of the usual carbohydrate substrates *N sicca* is also unpigmented but ferments glucose maltose fructose and sucrose this species is distinguished from the meningococcus by its more abundant growth and resistance its dry rough cohesive colony and antigenically *N pharyngis* is chromogenic, forming greenish yellow colonies and ferments carbohydrates with strain variations *N sicca* and *N pharyngis* appear to be antigenically distinct The generic name *Neisseria* probably should be restricted to oxidase producing species and strictly anaerobic organisms described under this name may more fittingly be placed elsewhere Langford et al (1950) found that all but 1 of 61 strains of anaerobic gram negative cocci isolated from the mouth or obtained from various collections could be classed as Veillonella the exception resembled a streptococcus

Veillonella A single species *V gazogenes* may be defined on the basis of distinctive properties This organism has been named *Micrococcus lactilyticus* by Foubert and Douglas (1948) who found it to be gram positive in very young cultures However, it is uniformly gram negative after 12 hours growth and appears sufficiently different in other properties to warrant retaining the older name which is widely accepted

V gazogenes occurs in saliva regularly and in high concentrations Douglas (1950) and Rogosa (1956) have described selective media for it *V ga ogenes* is a small mass forming spherical coccus strictly anaerobic principally distinguished by its unusual fermenta

strains of micro aerophilic actinomycetes from human and various animal sources by agglutination Both rough and smooth variants of *A israeli* whether isolated from actinomycotic lesions or from mucous membranes have been found to be capable of producing actinomycosislike lesions in small experimental animals

The degree of anaerobiosis required by actinomycetes is known to vary with different strains and has not been clearly correlated with either natural or artificial pathogenicity *A israeli* is a variably anaerobic nonmotile nonsporulating gram positive non acid fast bacterium Its rough varieties which show characteristic white opaque heaped up irregular colonies on agar and grow as crumbs at the bottom of otherwise clear broth are highly characteristic but smooth varieties are easily confused with diphtheroids or lactobacilli With some variation most students of this species have found it to have the following distinguishing features *A israeli* is unpigmented nonhemolytic produces acid without gas from a varying range of carbohydrates reduces nitrate fails to liquefy gelatin or coagulate egg white or serum and does not contain catalase Most strains prefer and many require strictly anaerobic conditions Pine and Howell (1956) found that lactic acid is the primary product of glucose fermentation with smaller amounts of succinic acetic and formic acids

Leptotrichia buccalis This is a distinctive bacterium which thus far has been clearly identified only in the mouth of man and several animals where it occurs regularly on tooth surfaces on the dorsum of the tongue and in saliva Hamilton and Zahler (1957) confirming the findings of earlier workers have clearly established the status of this organism

L buccalis is a rather large nonmotile nonsporulating unbranched anaerobic or microaerophilic gram positive rod easily decolorized in older cultures It occurs singly often as rods with one end flattened and one blunt pointed in pairs with the flattened ends approximated or in filaments either straight and septate or regularly twisted in pairs like rope strands The surface colony is typically translucent with an irregular medusa head shape or round and coarsely convoluted The addition of soluble starch to the medium favors isolation Mueller Hinton agar is satisfactory when enriched with 10 per cent serum or ascitic fluid Glucose sucrose maltose and other carbohydrates are fermented with a final pH of 5 or slightly lower gas is not formed The strains of this species studied by Jackins and Barker (1951) induced a lactic fermentation and did not attack amino acids *L buccalis* does not liquefy gelatin or produce indol and does not reduce nitrate H_2S is either not formed or may occur in trace amounts Catalase is not present

Mycobacterium *M smegmatis* was isolated from preputial and labial accumulations at the end of the 19th century and is considered as a valid species by Gordon and Smith (1953) (See Chap 11)

Spore bearing Bacilli Although species of both *Bacillus* and *Clostridium* have frequently been recovered from human skin and mucous membrane sites their irregularity in most of these areas and the ubiquity of both genera in soil and other environmental sites suggests that they be regarded as nonindigenous However *Clostridium* species have been found in human feces so regularly that they merit listing here *C perfringens* (type A) seems to be invariably present *C tetani* has been recovered in from 2 to 35 per cent of specimens examined by different workers Proteolytic clostridia have been found in feces irregularly

Gram negative Aerobic Bacilli Only *Escherichia coli* appears to be unequivocally indigenous and numerically prominent principally in the lower intestine Serologic typing of *E coli* has distinguished between resident and transient strains both of which may be found at any one time the former predominating Other bacteria in this group that appear generally to be saprophytes but are frequently recovered especially from feces as well as from pathologic sources include the *Klebsiella Aerobacter* group *Escherichia freundii* *Proteus* (*mirabilis* less often *morgani* and *vulgaris*) *Pseudomonas aeruginosa* and *Alcaligenes faecalis* That these organisms may occur in the intestine in low concentrations more often than is recognized is suggested by the frequent emergence of some of them following treatment with antibiotics and the apparently widespread occurrence of hemagglutinating antibodies to *P aeruginosa* (Gaines and Landy, 1955)

In more recent years it has been made clear that apparently indigenous diphtheroids having all three fermentation patterns with variations in other respects that have not provided a basis for classification occur in the pharynx and also in the vagina Other body surfaces do not appear to have been examined for these species The majority of Laughton s (1950) vaginal cultures were xeroselike in fermentation reactions but many of these resembled *C hofmannii* or *C diphtheriae* in morphology and staining properties Some of the xeroselike strains were actively acidogenic were said to occur in the premenopausal vagina even more commonly than Doderlein s lactobacillus and were thought to play a definite role in producing the low vaginal pH usually attributed to the lactobacillus It may be noted that the animal pathogen *C pyogenes* which differs from *C xerose* principally in that the former is hemolytic and liquefies gelatin may lose these distinguishing features under cultivation and thus come to resemble the actively acidogenic variant of *C xerose*

AEROBIC DIPHTHEROIDLIKE BACTERIA These bacteria have been rather widely reported as occurring in certain routine blood cultures under unusual conditions of cultivation—prolonged incubation blind subculture or incubation with 10 per cent CO The literature up to 1952 has been reviewed by Fleisher (1952) In many instances such positive cultures have been associated with disease of lymphoid tissue including Hodgkin s disease but the isolated organisms have not been found to be pathogenic The 50 strains studied by Carpenter et al (1956) had minute colonies were catalase positive failed to produce acetylmethyl carbinol but were variable in all other respects Specific names have not been assigned to these organisms It is not clear whether they are indigenous or as the findings of Kassel and Rottino (1955) suggest skin or air contaminants Their possible relationship to the anaerobic *Propionibacterium acnes* (see below) merits investigation

Anaerobic diphtheroidlike bacteria are known to be common both on skin and on mucous membranes but their taxonomic status is in doubt

Prévot s (1948) *Corynebacterium anaerobium* has been reported by Beerens and Dermont (1949) to be indistinguishable from *Actinomyces israeli* A micro aerophilic organism often called *Corynebacterium acnes* was renamed *Propionibacterium acnes* by Douglas and Gunter (1946) This organism seems to be the most numerous member of the biota of certain areas of skin and apparently is widely distributed elsewhere The findings of King and Meyer (1957) clearly indicate that the anaerobic diphtheroids that have been confused with *A israeli* are serologically indistinguishable from *P acnes* Douglas and Gunter found *P acnes* to be one of the principal contaminants of human blood plasma destined for a blood bank and suggested that it may enter the blood from skin via inadequate skin disinfection preceding venipuncture Gutierrez (1953) found what appears to be the same organism in the rumen of sheep and cattle and in hay and soil and regarded it as widespread in nature Thus the organism would appear to be a saprophyte like most species of a robic lactobacilli *P acnes* is variably anaerobic most strains prefer anaerobic conditions but may grow in air with a heavy inoculum its morphology varies from uniform short plump rods often in V shaped pairs under anaerobic conditions to diphtheroid clubbing and even rudimentary branching under aerobic conditions Gelatin is slowly liquefied a rennet curd followed by digestion of casein occurs in litmus milk catalase is consistently positive Douglas and Gunter observed β hemolysis on a buffered glucose agar containing 5 per cent of citrated human blood and noted a propionic acid fermentation of glucose Gutierrez reported fermentation of lactate in animal and saprophytic strains and stated that Douglas had also observed lactate utilization in strains from human sources

Actinomyces The only actinomycetes that appear to be indigenous to man are the anaerobic or micro aerophilic varieties which subject to further clarification may here be grouped in the single species *A israeli* These forms are found in the mouth the pharynx and the intestine and in the lesions of actinomycosis They appear to be strict parasites of man and many animal species A distinction between the smooth *A bovis* isolated from cattle and the rough *A israeli* from man may not be tenable It is recognized that both smooth and rough forms may be found in either host species and that rough strains may become smooth after cultivation Slack et al (1955) were unable to distinguish 20

medium *F fusiforme* can be recognized by a smooth surface colony in which whitish flecks appear by reflected light in a transparent matrix but rough colonies less distinctive also occur The cells are relatively small uniformly gram negative with both ends tapered to a point often occurring in roughly parallel bundles or sheaves They may be evenly stained or granular Tandem pairs are common as are filaments All these forms may show granules often regularly spaced in the cell or the filament *F fusiforme* is distinguished from *Leptotrichia buccalis* morphologically and in that the former is consistently gram negative yields a terminal pH in glucose of 6 or above and forms both H S and indol The cellular features of *F fusiforme* may be duplicated in *B funduliformis* but the former does not normally show the more bizarre swollen forms characteristic of bacteroides *F fusiforme* is closely related to *B funduliformis* biochemically and serologically According to Beerens (1953 54) *F fusiforme* can be distinguished from bacteroides by failure of the former to grow in 40 per cent bile while Guillaume et al (1956) reported the further distinction that *F fusiforme* consistently produces formic acid from glucose but little or no propionic acid while butyric acid usually appears in even higher concentrations in this organism Jackins and Barker (1951) found that *F fusiforme* produces NH_3 reduces NO_3 and attacks both amino acids and carbohydrates by a modified butyric fermentation

Motile Anaerobic Bacilli In fresh preparations from the gingival area tonsillar crypts feces or external genitalia examined under darkfield or phase microscopes motile bacilli are usually prominent because of their motility although they are seldom numerous compared with other bacteria

At least 4 species can be distinguished in the more recent literature *Fusobacterium girans* is a gram negative fusiform bacillus characterized by a slow gliding motility it is a strict anaerobe requiring enriched media forming variable colonies that may be smooth and elevated or rough with a characteristic agar depression Macdonald (1953) grew it in enriched veal heart infusion broth and found it to ferment glucose and sucrose and to acidify litmus milk neither H S nor indol was produced Macdonald et al (1953) and Berger (1956) have been unable to demonstrate flagella on this organism by either optical or electron microscopy *Bacteroides serpens* was originally described by Veillon and Zuber in 1898 and has been studied more recently by Steen and Thjøtta (1950) It appears to be similar to *B funduliformis* morphologically and biochemically but is described as peritrichously flagellated actively motile and capable of showing serpentine or spirochetelike forms as well as short straight rods and long filaments Steen and Thjøtta recovered it from a cerebral abscess grew it on ordinary blood agar and in glucose broth anaerobically but obtained better growth on addition of blood and cysteine H S was produced in glucose cysteine broth indol was also formed in broth after 5 days incubation but not in peptone water *Vibrio sputorum* is a small straight or curved gram negative rod with one or more terminal flagella characterized by rapid darting back and forth movement or by movement in a circular orbit It grows on enriched blood agar anaerobically as small grayish smooth colonies often surrounded by a zone of greening after exposure to air Growth in broth is poor carbohydrates are not fermented H S is formed but indol is not nitrate reduction is variable (Macdonald 1953) Moore (1954) recovered similar anaerobic vibrios from the vagina and reported good growth in the Dubos Middlebrook medium with the serum albumin concentration doubled Moore s strains were definitely crescentic showed up to 6 flagella attached terminally or laterally and had more erratic motility than tho e described by Macdonald They failed to form H S or indol or to reduce nitrate and did not ferment carbohydrates *Selenomonas sputigena* (*Spirillum sputigenum*) was probably both the vibriolike and the spiral bacterium described by Leeuwenhoek in 1683 It is found especially in the mouth in one or more of its various forms it is often con picuous by virtue of its large size and striking morphology and in fresh preparations by its motility It has been seen and occasionally cultivated by many workers but its status began to acquire clarity only with the work of Macdonald (1953) *S sputigena* occurs as a rather large vibriolike form with characteristically erratic tumbling motility as S shaped or helical forms or as large crescent shaped organisms with blunt points It is usually gram negative but may appear weakly gram positive This eems to be the so-called large fusiform bacillus so characteristic of smears from Vincent s angina Its flagella have been found by several workers to be peritrichous but they frequently appear on the concave side of vibrios or crescents

A number of poorly defined gram negative bacteria occasionally isolated from the healthy vagina or from urine the upper respiratory tract sputum or feces and from a variety of pathologic conditions include members of the tribe *Mimeae* (genera *Mima Herrellea* and *Colloides*) *Bacterium anitratum* and the B5W group (Stuart et al 1949) These are closely related organisms appearing coccoidal on solid media and in liquid media as bacillary or filamentous as well as coccal forms They are oxidase negative *Moraxella* (especially *M duplex* var *non liquefaciens*) and *Mima polymorpha* var *oxidans* are morphologically similar but oxidase positive Some or all of these forms (notably *B anitratum*) appear to be indigenous to man Members of the genus *Hemophilus* including *H parainfluenzae H hemolyticus* and *H parahemolyticus* have been isolated from the nasopharynx the nose and the mouth of healthy individuals

Bacteroides This generic term is restricted here provisionally to nonmotile nonsporulating strictly anaerobic gram negative bacillary or filamentous indigenous species It includes forms listed by others as *Necrobacterium* or *Spherophorus* and by misnomers such as *Actinomyces* (e g *A necrophorus*) Related motile forms are more conveniently considered apart *Fusobacterium* as will be noted is not clearly distinct from *Bacteroides* Bacteroides are most common and numerous in the lower intestine where indeed they have been found as the predominant species by Eggerth and Gagnon and others They also occur on other mucous membranes but much less prominently No adequate specific classification of this group can yet be made because of wide variation in all characteristics thus far studied

Many cultures are reported to grow on simple media after subculture but isolation is most successful on enriched media e g infusion agar containing 10 per cent of sheep blood under strictly anaerobic conditions Finegold et al (1957) recommended a combination of neomycin (200 mcg per ml) and Vancomycin (7 5 mcg per ml) in blood agar as a selective medium for bacteroides Many strains grow well in fluid thioglycollate medium preferably heated to expel dissolved oxygen and chilled rapidly before inoculation then ealed with solid petrolatum Initial cultures should be incubated 5 to 8 days at 37 after isolation many strains grow well in 24 hours On the assumption that forms isolated from pathologic processes in man and animals were derived from the mucous membranes, the following specific names may be given as representative of the group *B fragilis* is distinguished as having a smooth translucent colony and showing comparatively regular cellular morphology It is said to produce H S but not indol and to form gas in serum peptone water *B funduliformis* and *B necrophorus* seem to be closely related Both form either smooth or rough colonies and show highly pleomorphic cells with some regular rods or fusiform elements long filaments of varying width clubs and central (funduliform) swellings *B funduliformis* is the name usually used for forms isolated from man, *B necrophorus* for animal strains the latter is more often reported as pathogenic for mice or rabbits Beerens (1953) stated that *B necrophorus* can be distinguished by its ability to agglutinate chicken and sheep erythrocytes his strains otherwise were all weakly acidogenic in glucose maltose and galactose produced gas in Rosenow s medium grew in 40 per cent bile formed both H S and indol and failed to liquefy gelatin Jonsen and Thjotta (1948) reported acid and gas production from carbohydrates and formation of H S and indol but noted that earlier workers had obtained negative results for either or both of the latter substances *B nigrescens* the *B melaninogenicum* of earlier workers was renamed by Schwabacher et al (1947) who found its distinguishing black pigment to be hematin produced from hemoglobin not a melanin as had been assumed formerly This organism grows slowly and appears to have exacting nutritive requirements it is often difficult to separate from contaminating cocci or diphtheroids The pigment appears after growth for 5 days or more on blood media coincident with disappearance of hemoglobin from the surrounding medium Indol and H S are both formed

Fusobacterium fusiforme A single rather well defined species of anaerobic gram negative nonmotile fusiform bacilli can be distinguished although other varieties intergrading with *Bacteroides* seem to exist This species is found in saliva and on other mucous membranes

The studies of Bøe (1941) have been most important in establishing the status of *F fusiforme* It is best isolated on enriched media containing crystal violet Omata and Disraely (1956) have developed a useful selective

mucous membranes—it seems clear that variations of the indigenous biota from area to area are quantitative rather than qualitative and hence that the biota can be dealt with properly in a unified way. The areas of maximum concentration, the mouth and the lower intestine, are those in which pabulum is most abundant and concentrated and the biota is presumably *least subject to the antibacterial* effects of the host defenses. In both areas the bacterial concentrations as measured are those of micro-organisms in passage from the mouth

TABLE 54 DISTRIBUTION OF INDIGENOUS BACTERIA

	Skin	Conjunctiva	Nose	Pharynx	Mouth	Lower Intestine	External Genitalia	Vagina
Micrococcus albus								
epidermidis	++[3]			*		*		+
Streptococcus								
faecalis etc					+	7	+	+
hominis			*	+		+	+	
mitis				++		+	+	+
putridus micros etc				+	+	++	++	*
Neisseria catarrhalis			*	++	+		+	*
sicca pharyngis				+				*
Veillonella gazogenes					8		+	
Lactobacillus casei					0–5	+		
acidophilus					0–4	+		++?
fermenti					0–5	+		
brevis					0–4	+		
bifidus etc					+	8–10[4]		+
Corynebacterium								
hofmannii			+	++	+	+		+
xerose etc		+		+	+	+		++
Propionibacterium acnes	5[5]							*
Actinomyces israelii				+	+	+		
Leptotrichia buccalis					+			
Mycobacterium smegmatis etc							*	
Clostridium perfringens						4 5		
Hemophilus spp					*			
Moraxella spp								
Mimeae							*	*
Escherichia coli	*				0–3	7–8	+	
freundii								
Proteus mirabilis etc						< 6[5]		
Pseudomonas aeruginosa						*		
Klebsiella aerogenes				*				
Alcaligenes faecalis								
Bacteroides fundiliformis etc				+	++	9 10	+	
Fusobacterium fusiforme				+	5	+	+	
girans				+	+	+	+	
Bacteroides serpens				+	+	+	+	
Vibrio sputorum				+	+	+	+	
Selenomonas sputigena				+	+	+	+	
Treponema Borrelia				+	++	+	+	

Numerals = approximate average log counts per ml or Gm except as noted
Irregular or uncertain
+ Common or constant
++ Common and numerous

[1] Grubb and Puetzer (1947) have described a method for counting nasal bacteria total counts per ml in 20 ml of washings on aerobic blood agar ranged from 10 to 10^5 in 5 subjects species recovered were not noted
[2] For nonhemolytic streptococci and *M albus* in the pharynx see Kaplan Larkin and Hotz 1957
[3] Evans et al (1950) found an average of more than 5×10^5 *P acnes* per sq cm of skin in the scapular and deltoid regions as compared with 350 aerobes (mainly *M epidermidis*—*M albus*)
[4] In infant feces see Gyllenberg and Roine 1957
[5] See Ruebner 1957

This feature is emphasized by Lessel and Breed (1954) and depicted in the addendum to their paper by Robinow It is given by the former workers as a basis for applying the generic name *Selenomonas* Good growth occurs in fluid thioglycollate broth under solid petrolatum but not on agar surfaces The organism is strictly anaerobic ferments glucose and sucrose with a final pH of 5 1 5 3 reduces NO_3 but fails to form H_2S or indol Macdonald and Madlener (1957) have described an agar medium on which *S sputigena* grows as a spreading film which permitted successful isolation in 16 out of 21 trials

Spirochetes are among the most prominent of indigenous micro organisms because of their distinctive morphology and motility They occur characteristically in the gingival areas of the mouth in the tonsillar crypts in the lower intestine and on the external genitalia They have been grown in vitro apart from bacteria by many workers beginning with Ellerman in 1904

The procedure most often and most successfully employed is that used by Noguchi in 1912 which has been modified and simplified by later workers This procedure in which spirochetes grow into and may be subcultured from an agar medium at a distance from a core of mixed bacterial growth eliminates bacteria but does not yield separated colonies of spirochetes and hence gives no assurance that such bacteria free cultures are pure in the accepted sense Morphologic variability is such that any of the forms observed in source material (and additional variants never seen under such conditions) may sometimes appear in a single culture Attempts to classify cultures by biochemical and serologic means have also revealed a bewildering heterogeneity Consequently it is not possible with any assurance to decide whether all the indigenous spirochetes belong in a single variable species on the one hand or in the multitudes of species to be found listed in the older literature Confusion among indigenous spirochetes is compounded by the common practice of attributing specific status to forms seen in or cultured from different indigenous loci e g the mouth the lower intestine or the genitalia This practice if we may judge it by the distribution of other bacterial types is probably indefensible Moreover it may be assumed that the mouth is the original portal of entry and the primary site from which the spirochetes of all the other areas are derived Hence if specific designations are to be offered tentatively those commonly applied to oral spirochetes may be chosen Three such designations are representative of the commonest morphologic varieties *Treponema microdentium* the small closely wound pallidumlike spirochete most commonly seen in closed fusospirochetal lesions (e g lung abscess in man experimental abscesses or spreading lesions in guinea pigs) and also appearing most frequently in cultures *Borrelia vincentii* the loosely wound form single contoured under darkfield illumination, and *B buccale* the loosely wound double contoured form Contrary to a common impression based on fixed films all these spirochetes when actively motile are regularly wound Intermediate forms between the three as noted occur as well as forms resembling leptospiras occasionally with hooked ends

DISTRIBUTION OF THE INDIGENOUS BACTERIA

Table 54 lists the species and the classes of bacteria other than known pathogens as described in the preceding pages by their distribution in the various human body areas or surfaces Rough quantitative data are given where they are available principally for the mouth (saliva) and the intestine (feces) The skin is treated as a single area in the table although it is probable that different skin areas show as many characteristic variations in their indigenous biota as different mucous surfaces More exposed skin areas such as scalp face neck and hands and in particular retentive sites like fingernails and external ear are especially subject to environmental contamination as well as to contamination from the indigenous biota itself and the effort to separate indigenous from saprophytic species among those recovered from these sites is particularly difficult The microbiology of the external ear with special reference to aerobic forms and fungi has been reviewed by Senturia (1957) Skin bordering mucous orifices is likely to show the corresponding mucous membrane biota Evans et al (1950) selected a skin area in the scapular and deltoid region in an effort to identify the indigenous biota of skin and the data in the table reflect their findings

If the suggestion noted previously be accepted provisionally—that the predominant bacterium of skin *P acnes* may also occur on

in activities favorable to man. Nevertheless, if one rejects as fruitless the idea that the indigenous biota or any member of section of it is inert, it must be admitted that we are largely ignorant of the roles played by most of the indigenous species and that as yet we cannot formulate any useful generalizations with respect to the role or roles played by the biota as a whole. Most of the available information, as would be expected, relates to disease and will be dealt with under that head; data concerning activities other than disease are for the most part inferential; large stretches of this territory are virtually unexplored.

ACTIVITIES OTHER THAN DISEASE

Studies of animals reared and maintained under "germ free" conditions would be expected to provide fundamental information on the significance of the indigenous biota. Thus far however, apparently because of the technical difficulty and the high cost of such studies, their useful fruits are few. Chicks, monkeys, rats and guinea pigs have been reared in the absence of cultivable microorganisms. Guinea pigs appear to have done poorly, while among mammals the rat has been handled most successfully. In this species it seems clear that an indigenous biota is not essential for survival and reproduction. While it appears that germ free animals have more stringent requirements for vitamins in the diet than do control animals raised in the usual way—and even that the former may require unknown vitamins ordinarily synthesized by the intestinal biota—these points are not established beyond question (see Gustafsson, 1948). Reyniers and his co-workers have suggested, on the contrary, that the presence of vitamins in the cecum of germ free chicks indicates that they may be synthesized without the intervention of bacteria. Wagner (1955) reported that the serum of germ free chicks did not show the antibodies to intestinal bacteria found in normals although both groups responded to immunization. Gordon (1955) found that 8th generation germ free rats appeared to be normal in all respects except that their gastro-intestinal tracts and associated lymph nodes weighed less than in control animals; the lymphocyte content of the ileocecal tonsil in the former group was one tenth that of the controls. Appropriate exposure of germ free animals to contaminants led to a rapid increase in lymphocytes. Reyniers (1946) has noted that "when a germ free animal is suddenly brought to the outside without exercising care as to the contaminations with which it comes in contact, it usually dies," but the implication that saprophytes or ordinarily nonpathogenic indigenous species may be pathogenic in such instances has not yet been made clear.

However, it may be inferred from other data that the indigenous biota serves the symbiotic functions of aiding in the digestive process, synthesizing vitamins, and inducing the formation of antibodies which serve to protect the host against invasion by the biota itself. Bacterial participation in the digestive process has been studied in ruminant animals, while vitamin synthesis has been investigated in the rat and the human intestine as well as in the rumen. It is known that *E. coli* and other enteric gram negative aerobes synthesize B vitamins, as would be expected from their inexacting nutritive requirements, and that such synthesis is in excess of the requirements of the bacterial species. Direct evidence of synthesis of thiamine, riboflavin, nicotinamide and nicotinic acid in the human intestine has been obtained from feeding experiments. Interference with such synthesis seems to form part of the pathogenesis of the perlèche-thrush-diarrheal syndrome that may follow oral administration of chemotherapeutic agents, complicated by *Candida albicans* infection. It is also of interest that growth stimulation of animal induced by incorporation of antibiotics in commercial feeds has been attributed both to stimulation of vitamin synthesizing bacteria, e.g., *E. coli*, *A. aerogenes* and *Proteus*, and to inhibition of vitamin requiring forms, e.g., lactobacilli. On the other hand, plant growth stimulation by antibiotics under aseptic conditions indicates that the effect in this instance is independent of micro-organisms.

Circulating antibodies to indigenous bacteria have been looked for irregularly *in man*, usually in relation to disease, but it is noteworthy that they have been found in numerous instances, e.g., to greening streptococci, lactobacilli, actinomycetes, *E. coli*, *Ps. aeruginosa*, bacteroides and spirochetes of the *T. micro*

to the lower alimentary tract and from the intestine to the external world the data as they are collected do not necessarily reflect the true concentrations that reside upon the mucous surfaces themselves In the mouth however the values are usually based on saliva collected in interprandial periods and it is known that even when studied after brushing the teeth and rinsing the mouth the total salivary concentration is reduced by only some 50 per cent While equivalent data for other areas are scarce it seems likely that their concentrations are all very much lower For example the biota of the small intestine has been described as sparse and apparently similar in distribution of varieties to that of the mouth Mucous areas not noted in the table e g the esophagus the larynx and the trachea and the external portion of the male urethra appear to contain micro organisms derived from adjacent mucous or skin surfaces while the stomach and the deeper respiratory and genito urinary surfaces seem to be either potentially sterile or to contain only transient micro organisms

SOURCES OF THE BIOTA

The indigenous bacteria appear to be derived not from the external world at large but principally from the biota of other persons The skin is contaminated at birth from the maternal vagina The mouth is the first mucous area to show micro organisms—within 6 to 10 hours after birth—and aided probably in small part by the nose becomes the most important primary source of the biotas of all other areas The infant mouth in its role as a principal organ for transmission of information about the external world as emphasized by Freud must aid in the development of the oral biota and subsequently seed all the other areas In early life however the mouth contains principally aerobic forms the adult biota is conditioned in large part by the gingival crevice and therefore its full development awaits eruption of the teeth That the biota is maintained by continual contamination is suggested by its reversion to type after cessation of antibiotic therapy at any age In the intestine bacteria appear toward the end of the first day after birth In the breast fed infant there i an early phase of developing infection (Tissier, 1900) during which white staphylococci, colon bacilli spore bearing anaerobes, enterococci and other bacteria appear in increasing numbers and then disappear rapidly In the "phase of transformation *Lactobacillus bifidus* comes by the third or the fourth day to constitute as much as 99 per cent of the total organisms of the feces In bottle fed infants the phase of developing infection is more prolonged reaching its peak toward the fourth day there is no well marked transformation and while the *L bifidus* concentration may be equally high it is overshadowed by equivalent or still higher concentrations of coliforms and other bacteria A comparable transformation occurs in the vagina Aerobic lactobacilli appear at about the third day after birth, apparently favored by the strong acidity of the neonatal vaginal secretion and persist for a few weeks in association with a simple biota Thereafter as the secretion becomes scanty and more alkaline the biota becomes more varied and predominantly coccal At puberty there is a sudden reversion to an acidogenic group of species that appears to consist of both aerobic and anaerobic lactobacilli diphtheroids and yeast like fungi associated with deposition of glycogen in the vaginal wall apparently as the result of ovarian activity The low pH of the secretion resulting from fermentation of glycogen is looked upon as a defense mechanism that prevents establishment in the vagina of foreign and possibly harmful bacteria After the menopause the prepubertal scanty alkaline secretion and varied biota return

SIGNIFICANCE OF THE INDIGENOUS BIOTA

During the emergence of microbiology in the second half of the 19th century it was probably inevitable that the indigenous biota should have come to be dismissed as ' normal or 'harmless hence as unimportant or uninteresting In that interval as Dubos (1955) has pointed out immediate practical problems of disease necessarily became the focus of the effulgent new science with understanding and control of disease its prime objective Today the focus of medical microbiology is shifting even though its objective remains the same In the third decade of the antibiotic era it is becoming clearer that the indigenous biota may participate both in significant disease and

(talin_) pecies similar but not identical findings have been interpreted as dependent *on depletion of nutrients or* as induced by labile diffusible antibiotic substances The production of antibiotics or inhibitory states by indigenous micro-organisms has been widely observed *but its significance in vivo* can only be surmised Rosebury et al (1954) proposed a rou hly quantitative screening procedure for interactive phenomena among pairs of cultures grown *from drop* inocula on agar and attempted to classify such phenomena Predominance of lactobacilli in the vagina and pre umably in the nurseling inte tine has been commonly attributed to the low pH produced by these form and tolerated by them but by few others Phenomena involving known or presumed antibiotic substances as such and occurring in more varied *biotas* e g in the mouth or the adult lower intestine are evi dently more ubtle The characteristic domi nance of nonhemolytic streptococci among the aerobic bacteria of the mouth and the throat may be explainable in part by the inhibitory effects of the e forms against a wide range of other species including both saprophytes and pathogens as well as indigenous pecies Part of this effect is evidently due to hydrogen peroxide produced by the streptococci On the other hand some of the antibiotic effects of indigenous streptococci e g their antagonism *of S pyogenes* which appears approximately equally under aerob c and anaerobic condi tions are apparently independent of peroxide Th latter phenomenon (which like the inhi bition of *C diphtheriae* by *S mitis* has also been shown to affect animal pathogenicity) is a mutual antagonism in which either species is inhibited in the presence of a high concen tration of the other

Another group of antagonistic phenomena is concerned with *E coli* as inhibitor and may underlie in part the prominence of this species in the intestinal biota Inhibited pecies in clude *Candida albicans Proteus mirabilis Vibrio cholerae* and *Shigella* and *Salmonella* pecies Effects of *E coli* on other gram negative aerobes include some that appear to entail mutual antagonism comparable with the *S mitis—S pyogenes* interaction mentioned above *The possible influence of such phenom* ena on implantation of pathogens in the throat or the intestine seems to be worth considering Also included in the latter group are the *coli cines* (reviewed by Fredericq *1957*) These are a varied group of high molecular weight proteins or polypeptides produced by *E coli* and also by many *Shigella* and certain strains of *E freundii* and *Salmonella* acting specifi cally on strains of the same or related bacteria *including E coli* itself The colicines appear to bear some relation to bacteriophages but they do not app ar to contain DNA they kill sensitive bacteria without themselves *being replicated*

Antibioti substances are also produced by certain micrococci active against *C diph theriae* streptococci enteric pathogens and *other bacteria Halbert et al (1954) ob erved* that a group of strains of micrococci from the ocular biota inhibited *Clostridium septicum Corynebacterium hofmannii* and a test strain of *M albus* Micrococci that *inhibited C sep ticum* in vitro al o protected mice in experi mental mixed infections It is probable that many other interactive effects of significance *to* the indigenous biota await discovery *or* redi covery

PATHOGENIC EFFECTS OF THE INDIGENOUS BIOTA

Endogenous Infective Diseases Some General Principles

The subject to be urveyed here is so varied that no attempt can be made to cover it com pletely Both the idea that indigenous micro-organisms may *participate* in disease in the presence of predi po ing conditions and the term applied to such disease endogenous in fections seem to have originated with Esche rich in 1889 Yet although the idea has found support in relation to particular diseases and seems never to have been abandoned it has had a discontinuous history The apparent dif ficulty that such processes violate an unstated corollary of Koch s first postulate—the alleged causative agents being found in health as well as *in disease* and difficulty with the *third* postulate—isolated pure culture are nearly always nonpathogenic under cu tomary test conditions—may have aggravated a neglect based principally on the ground that until recent years there always seemed to be more urgent problems of disease that were al o for the most part less forbiddingly complex Today these obstacles no longer block the road the carrier state and silent infections negate as a fallacy the assumed corollary to the first postulate fulfillment of the third postulate has been accomplished often enough to establish the principle and the failure of the disease processes involved to show signs of

dentium variety It seems likely that the surface micro organisms are introduced into deeper tissues continually and give rise to specific antibody responses in doing so they may pave the way for allergic disease but they may also help to protect the host against their own more massive invasion

The idea is now widely credited that the indigenous biota maintains a balance both within itself and in relation to man such as to be compatible with or even contributory to health and that certain disturbances either of the host or within the biota may lead to disease Among the many phenomena contributed to this balance by the host those associated with pituitary adrenal hormones and with tissues injured or physiologic processes impaired by ionizing radiation have been studied particularly in recent years Administration of cortisone or ACTH or exposure to X radiation or both together have been found experimentally to lead to invasion by the intestinal biota particularly by *E coli* and other gram negative aerobic bacilli although whether these forms are actually most concerned or are selected by conventional aerobic cultural technics is not clear In mice and rats cortisone administration has been found to activate an apparently endogenous infection with *Corynebacterium pseudotuberculosis* but coliforms *Proteus* and *Pseudomonas* have also been found to be invasive in mice under similar conditions Whole body X irradiation of mice in the dosage range 450 to 700 r has been shown to lead rapidly to invasion of liver and spleen and later to bacteremia involving members of the aerobic gram negative intestinal biota Similar effects in mice have been produced with fast neutrons in the lethal range Several workers have reported that mortality in irradiated animals can be controlled at least in part with antibiotics Clinical data on atomic bomb casualties in Japan suggest that in man the fusospirochetal biota may participate in postirradiation disease

However in addition to these and other host conditions the development and the maintenance of the quantitative characteristics of the biota of each site are probably determined within the biota itself by a complex of co operative and competitive phenomena of species interaction Our understanding of the ecology of the indigenous biota as it is governed by all these circumstances is at present rudimentary Obviously, the problem can not be approached by studying pure cultures however the study of culture mixtures is a formidable undertaking Only a few examples can be given here

Co operative bacterial interactions generally entail the sharing of enzymatic mechanisms of dissimilation or synthesis or both by two or more species to yield products distinctive for the combination Additional phenomena may be assumed to occur such as the provision by aerobic forms of reducing conditions permitting the growth of anaerobes and genetic interchange between closely related bacteria in which transformed, recombined or transduced varieties emerge with new properties Examples of biochemical co operation include in addition to the classic satellite phenomenon involving *Hemophilus influenzae* and *Micrococcus aureus* satellite growth of *H parainfluenzae* due to *S pyogenes* stimulation of growth of *E coli* by *Candida albicans* and of *Bacteroides nigrescens* by cocci or diphtheroids Synergistic or symbiotic dissimilation include the production of gas from mannites by a mixture of *Proteus morgani* and *Shigella dysenteriae* Flexner and combined growth with synthesis of amino acids and vitamins of mixtures of *Lactobacillus* and *Leuconostoc* species with *S faecalis* on media inadequate for pure culture growth as reported by Nurmikko (1954 1955) and others Schultz Haudt and Sherp (1955) found that *F fusiforme* produces a phenolsulfatase adaptively when grown in mixtures with staphylococci or other bacteria The roles played in the indigenous biota of man by the anaerobic lactate fermenters *Veillonella gazogenes* *Propionibacterium acnes* and possibly *Selenomonas sputigena* may also find a place in this category in man as they have in ruminants

Competitive interactions would seem to be based on either or both of two general principles the removal from the environment by one species of one or more substances or conditions needed for survival or multiplication of other species or the production by one species of toxic or inhibitory substances or states It is curious that the former mechanism insofar as interactions between species are concerned has been difficult to establish The phenomena of staling or direct antagonism in which a test species is seeded into or streaked on a medium containing an old culture of another seems to depend on viability of the inhibiting

gens indigenous species and occasional saprophytes appears Occasional blood cultures show more than one species A significant proportion of clinically typical cases yield negative blood cultures such cases have a particularly high mortality rate Positive cultures have been recovered from bone marrow when blood cultures were negative It is recognized that a bacteremia may not be disclosed by routine culture methods Several workers (see Finland 1954) have emphasized the need for special methods for fastidious anaerobes

Pre existing cardiac damage most commonly affecting the mitral valve is an important antecedent of s b e In approximately 75 to 90 per cent of instances in which such damage is recognized it is rheumatic in origin in the remainder it may be arteriosclerotic congenital or syphilitic In many instances of rheumatic valvular injury the patient is unaware of the defect before the onset of s b e

The streptococci most frequently implicated in this disease and many of the other species recovered from blood cultures are entirely nonpathogenic for laboratory animals by the usual routes of inoculation However progressive disease closely similar to s b e in man has been produced experimentally in animals by a variety of means the most significant of which entail cardiac damage induced before intravenous inoculation of nonhemolytic streptococci This result has been accomplished by traumatizing the aortic or mitral valve in dogs with a long wire inserted into the left carotid artery and a month or more later injecting nonhemolytic streptococci intravenously Similar effects have been obtained in dogs by inoculating greening streptococci after surgical production of an arteriovenous shunt Highman et al (1952) showed that cardiac damage could be induced in rats exposed intermittently for prolonged periods in a low pressure chamber to simulated altitudes of 25 000 feet Some of these animals developed spontaneous bacterial implants Repeated intravenous injection of *S mitis* or a single large dose of *S faecalis* yielded bacterial endocarditis in a majority of the animals In the *S faecalis* injected animals penicillin was effective in controlling the infection if started 12 to 20 hours after inoculation but less so when delayed for 4½ days

The connecting link between such animal experiments and s b e in man is recognized to be a bacteremia usually transient whose inciting event is nearly as diver e as the microbic agents of the disea e With indigenous microorganisms the mechanism may include trauma manipulation or massage of a mucous or skin surface Tonsillectomy and dental operations have been particularly incriminated Determinant dental operations include tooth extraction and in patients with periodontal disease gingival instrumentation digital massage tooth brushing and even the chewing of hard candies but cavity preparation and pulpotomy were found by Beechen et al (1956) not to produce bacteremia in children Most reports agree that such bacteremias can be demonstrated within 10 minutes after the manipulation but not subsequently *Serratia marcescens* applied to the gingival area before tooth extraction has subsequently been recovered in peripheral blood

Even with increased awareness in recent years of the significance of such events the attempt to relate them to the development of s b e in individual patients has been successful only in some 35 per cent of instances Ernstene et al (1951) and Cates and Christie (1951) noted a history of tooth extraction in only 8 and 9 per cent respectively of their series of cases but agreed in listing this operation as the most frequently recorded precipitating agency Kelson and White (1945) estimated the risk of s b e after extraction of a tooth in a patient with cardiac disease as 1 in more than 500 These observations lend little support to suggestions that extraction of all teeth be recommended for prophylaxis of s b e in susceptible persons

The specific therapy of s b e has been reviewed by Finland (1954) and Beeson (1955) Current practice for patients with cultures positive for streptococci favors penicillin or a combination of penicillin and streptomycin The agent should be isolated and sensitivity tests done where possible before therapy is instituted 5 to 6 blood cultures are recommended at hourly intervals before therapy and daily for several days thereafter with cultures made by a variety of method and held for 2 to 3 weeks Recent practice favors moderate penicillin dosage for shorter periods than were used formerly and the use of penicillin even in allergic patients the allergy being controlled by other drugs Penicillinase has been

disappearing in the age of antibiotics leaves them with greatly increased relative if not absolute importance Before presenting a few examples of endogenous infective diseases it may be useful to suggest certain features that seem to be common to the whole group It has been pointed out already that diseases associated with indigenous micro organisms cannot be sharply distinguished from exogenous infections in which latency or its equivalent is a conspicuous feature Indeed it may be noted that the very word endogenous' is used only for convenience and must not be taken too seriously The group differs from endogenous reinfection in tuberculosis for example principally in that *M tuberculosis* is easily recognizable as a pathogen The bacteriologic revolution established the guiding rule that all disease strictly speaking is exogenous in that its 'causes or "determinants' are extrinsic and therefore amenable to control The group of endogenous infections so called is not an exception in this respect The common features of endogenous infective diseases may be listed as follows (1) Micro organisms found in the indigenous biota are indispensable agents in the pathogenesis of endogenous infective diseases (2) The microbial agents of endogenous infective diseases occur in the indigenous biota in health but in disease they are either significantly increased in concentration in or near their usual sites or are found proliferating in an unusual site—in the tissues (3) The bacterial agents of endogenous infective diseases are characterized by low intrinsic pathogenicity (4) A state equivalent to latency in exogenous infections is characteristic of endogenous infective diseases which therefore have no definable incubation period (5) Endogenous bacterial diseases are not communicable within the accepted limits of meaning of this term (6) Immunity in the sense of specific protection against recurrence of a given endogenous infective disease is not recognized clinically rather the diseases have a marked tendency either to recur or to progress slowly over a period many years (7) In endogenous infective diseases causes or "determinants other than the activity of indigenous microbic agents are as indispensable to pathogenesis as are the agents themselves Another feature of this group of diseases, frequent but apparently not universal, is that they are likely to be nonspecific with respect both to their microbic agents and to other determinants of pathogenesis It is usually futile to search for single ' causes of diseases in this group, or otherwise to attempt to deal with them by analogy with disease outside the group

SUBACUTE BACTERIAL ENDOCARDITIS

Subacute bacterial endocarditis (s b e) is in the majority of instances a fully characteristic endogenous infection whose pathogenesis is rather well understood and comparatively simple Implantation on a previously damaged heart valve of nonhemolytic streptococci or other micro organisms follows an initial transitory bacteremia induced by surgical or comparable manipulation The organisms proliferate in and under cover of fibrin platelet thrombi which protect them from phagocytosis and other blood clearing mechanisms forming vegetations which lead to persistent bacteremia and in untreated cases, to death by occlusion of the affected valve other vascular accidents or serious embolism The microbic agents concerned are usually nonpathogens pathogenesis seems to require of them only an ability to proliferate under conditions provided by the host

The earlier literature on s b e has been reviewed by Rosebury (1944) The disease occurs principally in the age range 15 to 35 years in both sexes but may appear at any age over 5 years Cates and Christie (1951) who reported on 442 cases in England and Wales noted that the incidence in those countries has remained at about 1 000 cases per year since s b e was distinguished from other fatal endocardial infections in 1931 In this country the disease continues as a serious problem Before chemotherapy the case fatality rate approached 100 per cent With penicillin treatment the death rate has dropped to 25 to 30 per cent A persistent bacteremia is characteristic but not invariable Approximately 90 per cent of positive blood cultures show oral or pharyngeal streptococci mainly the heterogeneous *S mitis* including forms occasionally hemolytic belonging to Lancefield groups other than group A Enterococci are recovered in approximately 4 per cent of additional cases In the remainder a diverse list of patho

violet dye in sections stained by Gram's method but may otherwise stain irregularly. Around the periphery the ends of individual filaments may project with or without radially arranged hyaline clubs. The clubs, when present, take the eosin stain and are several times larger than the filaments whose ends they enclose.

The epidemiology of actinomycosis is that of an endogenous infection as was clearly shown by Wolff and Israel in 1891 and by Wright in 1905. Nevertheless the pathogenesis of actinomycosis is incompletely known. In the hands of earlier workers attempts to demonstrate pathogenicity of *A. israeli* for common laboratory animals by the usual routes were usually unsuccessful or yielded only localized lesions.

Repeated passage, trauma during inoculation or admixture with other bacteria were generally ineffectual. However, progressive and fatal experimental actinomycosis with typical granules and other pathologic signs has been produced—although again without regularity—by repeated inoculation at intervals that might have permitted development of allergy to the organism, but allergy could not be demonstrated convincingly. Meyer and Verges (1950) and Geister and Meyer (1951) have reported the consistent production of actinomycosis in mice by inoculation of pure cultures with gastric mucin. Hazen et al (1952) produced typical actinomycosis in 21 of 28 young male hamsters with any of 8 strains of *A. israeli*. Gale and Waldron (1955) found that actinomycotic lesions with typical granules usually without clubs could be produced in mice with any of 7 strains of *A. israeli* but that the disease developed during the first week after inoculation and then tended to recede. Guinea pigs inoculated with a single strain responded similarly. These results may help to explain the failure of other workers who depended on gross illness or death as a sign of experimental infection. Strains of *A. israeli* isolated from mucous membranes have been found as capable of inducing lesions as strains obtained from the natural disease. The experimental findings as a whole indicate that *A. israeli* is an important determinant of actinomycosis but they leave the pathogenesis of the progressive disease in man uncertain. The comparative rarity of actinomycosis suggests that autoinoculation resulting from minor trauma, perhaps even when repeated, can be no more than a contributory incident. More severe traumatic events have often been associated with actinomycosis, e.g., a tooth extraction or other injury to mouth or throat, human bite or knuckle injuries from a blow to the teeth, or aspiration of an extracted tooth or tooth fragment into the lungs. *A. israeli* has been found in salivary calculus and detached masses of tartar may be involved in comparable traumatic accidents. Actinomycotic pus from closed lesions frequently, perhaps invariably, contains other organisms in addition to *A. israeli*; the suggestion has been made that a mixed infection may be important in establishing the disease.

Although the finding of microscopically typical "sulfur granules" is presumptively diagnostic of actinomycosis in man, the diagnosis cannot be considered as established unless *A. israeli* is isolated from the lesions. For this purpose plates should be serially streaked on brain heart medium containing 2 per cent agar or on blood agar and incubated anaerobically with 5 per cent CO_2 for from 4 to 6 days at 37°. The opaque white heaped-up colonies of *A. israeli*, particularly the rough colonies, can be identified easily even in the presence of abundant contaminating growth, but smooth colonies may be indistinguishable from those of diphtheroids. Isolation may be made into tubes containing a 10 cm column of glucose infusion broth in which the organism typically grows after aerobic incubation as a crumblike or tiny cauliflowerlike mass in the bottom with the supernatant broth clear, but smooth strains may grow more diffusely. In glucose agar shake cultures incubated aerobically for 4 to 6 days the typical picture is that of whitish spherical or mulberry-shaped colonies of varying size up to 3 mm in diameter growing only in the depths of the agar, often with a dense zone of colonies 0.5 to 2 cm below the free surface. Recovery of an occasional *A. israeli* colony from materials subjected to direct contamination from a mucous membrane (e.g., in expectorated sputum) has no diagnostic significance.

Actinomyces israeli is sensitive to penicillin and to a range of other antibiotics in vitro (see McVay and Sprunt, 1953). Penicillin, tetracyclines, chloramphenicol and isoniazid have all been reported as effective with occa

suggested for this purpose (Minno and Davis, 1957)

Control measures usually emphasize the administration of antibiotics before, during and after surgery or other potentially precipitating manipulations in patients with cardiac damage Several studies of this procedure agree that it reduces but does not abolish bacteremias With the increasing tendency to limit the use of penicillin to cases of severe illness in which it is the drug of choice one of the broad spectrum antibiotics may be selected for such prophylaxis At longer range the prevention of s b e is tied to the prevention of rheumatic fever or where rheumatic or other cardiac damage is already present, to the avoidance of other endogenous infections insofar as this is possible including more than the usual attention to preventive dental care (see Rosebury 1944)

ACTINOMYCOSIS

A subacute or chronic usually progressive disease of the orofacial thoracic or abdominal tissues actinomycosis, produced by *A israeli* alone or perhaps in combination with other micro organisms is another clear example of endogenous infection In this instance however while host factors in pathogenesis seem to be essential to permit or enable the occasional invasion by a surface parasite, the nature of these factors is poorly understood Actinomycosis occurs in man cattle and other animals It is characterized by the development of indurated granulating swellings chiefly in connective tissue by suppuration usually of limited extent and by the presence in the pus or lesions of *A israeli* The disease develops over periods ranging from a few weeks to a year or more and may spread widely by contiguity sometimes pointing toward the skin and forming fistulae that tend to heal and reform elsewhere rarely pointing toward mucous or serous membranes The organism may be disseminated through the blood or in the lungs through the bronchi The lymphatic system is only rarely involved Bone lesions are uncommon in man

Actinomycosis is generally thought to occur in the cervicofacial region somewhat more frequently than in all others combined Such cases are seen most frequently in dental or oral surgical clinics Fisher and Harvey (1956) found that of 90 cases treated over a 25 year period 55 per cent involved the abdominal wall or viscera, 23 per cent were thoracic 13 per cent were cervicofacial and 9 per cent appeared in other areas The commonest *cervicofacial* lesions are seen on the cheek or submaxillary skin as indurated or edematous swellings often bluish or reddish in color with a tendency to form a series of irregular folds separated by furrows the healing area forming scars as new lesions develop *Thoracic* actinomycosis is found mainly in the lungs with the formation of abscesses and cavities which are usually small Extensive abscesses may be found in the bronchi and their rupture may lead to dissemination of the infection by way of the bronchial tree Actinomycotic pleurisy and empyema have been observed as has involvement of the heart and the pericardium *Abdominal* actinomycosis may be found in any organ but is most common in the region of cecum and appendix From here the lesion may extend with suppurative foci and the formation of fibrous adhesions to the abdominal wall where skin lesions may appear similar to those of cervicofacial actinomycosis Or the lesion may remain circumscribed forming a fibromalike mass The liver is commonly attacked and lesions of the genital tract are relatively frequent The stomach the small intestine and the kidney are seldom affected Rarely actinomycosis may be found in the anorectal area or the testis

The microscopic appearance of the lesions of actinomycosis varies from that of an acute abscess with an abundance of polymorphonuclear cells to the more chronic lesion in which proliferating connective tissue is the most conspicuous feature Commonly the picture includes necrosis with an abundance of leukocytes surrounded in turn by granulation tissue and a profuse formation of dense fibrous tissue It is thus not characteristic unless *sulfur granules* are present These are frequently lacking in either tissue or pus and when present particularly in lesions in man may not show typical clubs Club bearing granules may be found in several distinct diseases distinguishable from those of true actinomycosis in gram stained but not in routine hematoxylin eosin sections Details of the typical granule are seen best under magnifications of 400 diameters or more It may be roughly circular or irregular in outline or may consist of several colonies of different size and shape that have coalesced The granule is composed of a dense reticulum of fibrils which take the

animals used by the earlier workers may have been fed deficient diets The more significant findings relating to pathogenicity are to be found in studies with mixtures of pure cultures which have yielded positive results consistently when the mixtures contained organisms identified as or resembling *S evolutus* *S putridus* (or incompletely identified anaerobic cocci) *B funduliformis* *B fragilis* or *B nigrescens* Meleney (1931) Henthorne and McDonald (1936) and Steinhorn (1945) have all reported that mixtures of anaerobic streptococci with staphylococci or unnamed second species inoculated subcutaneously produced ulcerative necrotic or gangrenous lesions often very destructive and sometimes fatal in guinea pigs mice or other animals the individual cultures inoculated alone showed little or no pathogenicity Altemeier (1942) produced severe cellulitis with abscess formation ulceration or gangrene in guinea pigs with mixtures containing 4 to 6 pure cultures isolated from peritonitis in man The pure cultures by themselves were again noninfective for the most part Five of the 6 mixtures included either an anaerobic streptococcus or a bacteroides culture the other species studied included aerobic cocci coliforms *C hofmannii* and *Ps aeruginosa* Ryff and Lee (1946) found that a strain of *B funduliformis* isolated from necrotic stomatitis in a calf produced lesions independently when inoculated in 0 5 ml amounts into the labial skin of rabbits When 0 1 ml of this culture in itself innocuous was mixed with an equal volume of one of a long series of pure cultures of other species most of which were also nonpathogenic independently necrotic or suppurative lesions resulted in the majority of trials Among the ancillary nonpathogens were indigenous aerobic streptococci micrococci or corynebacteria *B subtilis* and *L bifidus* In additional experiments by these workers more severe lesions resulted in rabbits given deficient diets and then inoculated similarly with the bacteroides cultures mixed with an aerobic streptococcus or *B subtilis* The most extensive studies of this kind were reported by Hite et al in 1949 These workers used a group of cultures isolated from the female genital tract under either normal or pathologic conditions including *B funduliformis* *B nigrescens* and other bacteroides a strain listed as *Bact fusiformis* but not otherwise identified a group of anaerobic streptococci and other cocci not clearly characterized and the aerobes *S liquefaciens* *S mitis* and *M albus* Subcutaneous inoculation of mice with single pure cultures of the anaerobes again yielded minimal lesions or none the aerobes alone were nonpathogenic In pairs however comprising either two anaerobes or anaerobe plus aerobe inoculation of half volumes of each yielded lesions with many of the mixtures described as necrotizing often suppurative sometimes extending to the peritoneum and the viscera and in some instances leading to death of the animal The most significantly pathogenic mixtures contained anaerobic cocci with either *S liquefaciens* or the fusiform bacillus bacteroides with any of the aerobes or curiously *B funduliformis* with either *B nigrescens* or the fusiform bacillus It is of interest that mixtures of bacteroides with the anaerobic cocci were generally ineffectual Synergistic infection was also observed when *S liquefaciens* was mixed with heat killed *B funduliformis* but mixtures of the same streptococcus with extracts of the latter species prepared by grinding with sand digestion with trypsin or extraction with trichloracetic acid had little or no effect

In vitro antibiotic sensitivity tests with these organisms have been reported most recently by Garrod (1955) and by Lodenkamper and Stienen (1955) The former found *B nigrescens* *B funduliformis* and a fusiform bacillus to be most sensitive to penicillin but *B fragilis* was penicillin resistant All 4 species were also sensitive to oxytetracycline and chloramphenicol more so to the former erythromycin was effective against *B nigrescens* and polymyxin against the fusiform bacillus and *B funduliformis* Streptomycin and bacitracin were generally ineffective The German workers studied both bacteroides and anaerobic cocci separately and as mixtures Best results were obtained with the tetracyclines but resistant strains were found to nearly all antibiotics tested

Such clinical data as are available are generally consistent with these findings Tetracyclines and chloramphenicol have been used effectively penicillin streptomycin and sulfonamides have usually failed

FUSOSPIROCHETAL DISEASES

The group of diseases in which indigenous spirochetes appear as prominent components of a profuse mixed biota include some of the most widespread ills of mankind The early literature on fusospirochetal disease has been reviewed by Rosebury (1938) The group includes the periodontal diseases which are

sional failures High dosage for sustained periods is usually used, where possible accompanied by surgical measures

BACTEROIDES AND ANAEROBIC STREPTOCOCCI—MIXED OR SYNERGISTIC ANAEROBIC INFECTIVE DISEASES

A diverse group of diseases of animals and man has been associated for many years with bacteroides or anaerobic streptococci separately or together or mixed with other bacterial species Bacteroides were described in calf diphtheria in 1884 by Loeffler and isolated from the same disease by Schmorl in 1891 they were recovered from fetid and gangrenous suppurations in man by Veillon and Zuber in 1897 Veillon isolated anaerobic streptococci from the lesions of Ludwig s angina, perinephric abscess and bartholinitis in 1893 Schottmuller first cultivated anaerobic streptococci from the blood in 1910 while bacteroides septicemia was first described by Tessier and his co workers as recently as 1929 *The literature on the occurrence of these organisms* in disease has accumulated steadily and continues to do so in aggregate it leaves no doubt that these species perhaps occasionally alone more often together or in other bacterial associations are capable of inducing significant disease However the manner in which they are enabled to do so the factors in the pathogenesis of these diseases other than endogenous infection is virtually unknown and constitutes an important problem for further study

The clinical processes concerned may be classified in three groups of which the second may differ only in microbic origin from disease associated with pathogenic streptococci staphylococci or enteric aerobic bacilli In the first, anaerobic or micro aerophilic streptococci occur with other indigenous bacteria in a spreading gangrene of the skin or in wound infections In the second either anaerobic streptococci or bacteroides or both recovered in blood cultures with or without additional species are found in diseases beginning especially in the pharyngeal intestinal or genital *mucous membranes* and progressing to a septicemia or pyemia often associated with thrombophlebitis In the third group in which indigenous spirochetes are prominently associated with bacteroides, anaerobic streptococci and a profusion of other indigenous species—the so called fusospirochetal diseases—the lesions are usually inflammatory or destructive processes of the mucous membranes themselves or of directly adjacent tissues The last group is considered separately in the following section the first two are dealt with here as a unit The distinction between the three groups is not sharp Bacteroides have been associated as the predominant infective agent with chronic ulcerative colitis while the fusospirochetal biota has been implicated in a form of cutaneous ulcer occurring in the tropics It need hardly be emphasized that collectively *these diseases* occupy a distinctly higher level of complexity than the endogenous infective diseases considered previously and it is not surprising that *the role* of indigenous micro organisms in these processes has seldom been credited and is frequently disregarded or overlooked The importance of the problem may be gauged by the following figures for incidence of the *bacteroides anaerobic streptococcus group of* organisms in hospital cultures during the past 2 decades as reported by different workers of 5 180 specimens 200 revealed nonsporulating anaerobes approximately half of which were bacteroides 20 cases were observed during 4 years in one instance, 47 in 8 years in another and in others 47 cases in about 2 years 35 in 5 years and 239 in 8 years The record suggests that these organisms have been found among hospital cases whenever appropriate means have been employed to look for them

The role of anaerobic streptococci and bacteroides in endogenous infective diseases is made clear most convincingly by studies of their experimental pathogenicity and is supported by the results of antibiotic therapy in man The following discussion is limited to *these two topics For reviews of other aspects* of this subject see Lahelle (1947) Carter et al (1953) and Alston (1955)

In pure culture anaerobic streptococci have been found to be nonpathogenic for animals almost uniformly As for bacteroides on the other hand early workers had little difficulty *demonstrating pathogenicity* especially with strains isolated from animal disease but since about 1935 such tests have nearly always resulted negatively Dack (1940) suggested that

an aerobic diphtheroid The diphtheroid was thought to serve the non pecific function of supporting growth of *B nigrescens* None of the species considered es ential by D T Smith and Iroske and Sayers was present in the inoculum nor did any appear in the exudates from which the 4 species employed were reisolated The positive aspects of these findings are in line with those noted previously in experimental studies of bacteroides but their negative aspects as Macdonald and his co workers themselves suggested and as Berger (1957) has pointed out cannot be con sidered as demonstrating that neither spiro chetes nor typical fusiform bacilli participate in fusospirochetal infections

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The pattern of ideal relationships entails a complex equilibrium changing with time be tween functional stress upon the teeth occlu sal tooth wear and continued eruption of the teeth and apposition of cementum probably accompanied by a rootward shift of the ter minal epithelium of the gingiva Functional stress transmitted through the teeth maintains the integrity of the periodontal ligament and the alveolar bone and both structures hift and change with age to compensate for cusp loss and interproximal attrition Additional compensatory changes with age involve these tissues the dental arches as units and the temporomandibular joints Disturbances in this process which alter the ideal relation ships of the attached epithelium the gingival margin and the underlying bony support impair the hygienic mechanism whereby ideally functional movements prevent gingi val accumulations of gross bacterial pabulum such accumulations and the consequent infec tion aggravate the cycle of changes

The circumstances that induce periodontal disharmony are themselves varied and com plex *They may include any agency that leads* to impaired cell metabolism of the tissues con cerned nutritional or hormonal upsets in toxications or exogenous infections abetted by tooth loss or damage resulting from dental *caries or by faulty dental restorations* and doubtless also by the character of ingested food as it promotes or fails to promote healthy function and retention of food *residues* in addition to its nutritional quality Emotional

treated in the next section, the complex of ulceromembranous stomatitis or pharyngitis or equivalent disease of the external genitalia, and the extreme form of this process, noma, comparable putrid necrotic diseases of the respiratory and the intestinal tracts a form of tropical ulcer of the skin and similar lesions of other areas Fusospirochetal disease seems always to be superimposed on tissue damage induced by other agencies, including scurvy pellagra inanition or other nutritional disturbances agranulocytosis or radiation injury viral infections including measles and primary herpes simplex and probably more commonly complexes or constellations of factors exemplified in the periodontal disease group

Experimental fusospirochetal disease was produced first in rabbits by Veszpremi in 1905 and 1907 and since repeatedly in guinea pigs and other animals by many workers The lesions have been initiated with whole exudates from any of a series of fusospirochetal diseases in man and are regularly transmissible with the resulting animal lesion exudates inoculated by any of several routes Subcutaneous inoculation of guinea pigs the method most commonly used yields either large necrotic abscesses tending to evacuate and heal or a spreading cellulitis which either heals after belated localization or more typically leads to death in 2 to 7 days In the more severe infections the exudate is serofibrinous it is always foul and contains spirochetes in a complex mixture of other bacteria In animals sacrificed while moribund a cellulitis is found dissecting the fascial planes from the point of inoculation the viscera are somewhat congested and the adrenals are enlarged and hemorrhagic but no visceral infection is found In such animals as in comparable disease in man spirochetes have been found in vital tissue while other bacteria are limited sometimes sharply to the distal border of the necrotic zone Pathologic changes are those of acute inflammation in fatal infection the cellular response is disorderly or at times apparently entirely lacking The contained species in one guinea pig passage exudate (Rosebury et al 1950) in which the LD_{50} was estimated as 8×10^9 organisms can be identified as spirochetes of the *T microdentium* variety *Fusobacterium fusiforme* and atypical fusiforms both *Vibrio sputorum* and *Selenomonas sputigena* atypical bacteroides *S mitis* and incompletely identified anaerobic streptococci and a miscellany of gram positive bacilli all except *S mitis* appeared to be strict anaerobes From another passage exudate Macdonald et al (1954) isolated a similar range of species, but only atypical fusiform organisms were present, as was an unidentified motile gram negative anaerobic bacillus and *B nigrescens* *S sputigena* was not found A later study by these workers (Macdonald et al, 1956) indicates that one of the cultures was an aerobic diphtheroid

Attempts to reproduce transmissible fusospirochetal infections with recombined mixtures of pure cultures and thus to define the essential component species have yielded equivocal results up to the present The earlier studies, initiated by Kritchewski and Seguin in 1920 have been reviewed by Rosebury et al (1950) D T Smith in 1932 and Proske and Sayers in 1934 reported that a mixture of pure cultures of 4 anaerobes—*T microdentium* a fusiform bacillus a vibrio and a streptococcus—was the only effective combination among many tested Rosebury et al (1950) using the cultures previously noted and others were unable to produce typical or transmissible fusospirochetal infection with similar or other combinations These workers showed that typical infection could be induced by inoculation of cultures grown from whole exudate as a mixture through 10 successive culture passages without loss of infectivity hence that the infective mixture is cultivable in vitro as such An estimate of the dilution of the original inoculum entailed by this procedure was in line with other studies by these workers which suggested that a virus is not a component of the infective complex Successful recombination was accomplished by Macdonald et al (1954) who produced typical transmissible fusospirochetal infection with material taken from the 'hub of 'wheel plates' on which 16 cultures isolated from guinea pig exudate had been streaked radially to a common center spirochetes from a culture having been added at the hub In 3 of 9 trials in which the spirochetes and all or all but one of the spoke cultures grew under these conditions an emulsion of the mixture reproduced the typical disease In a subsequent study Macdonald et al (1956) using the wheel plate method recombined the same 16 cultures in various groups and arrived at the surprising conclusion that the minimum group capable of eliciting typical transmissible infection consisted of 2 strains of *Bacteroides* (including *B nigrescens*) an unidentified motile gram negative anaerobic bacillus and

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disturbances interrelated with metabolic upsets and with salivary function, stimulated by gross fusospirochetal infection directly contributing to periodontal disease by clenching and grinding of the teeth and to neglect of nutrition and toothbrushing must also be included among the forces leading to periodontal disease These various agencies in different combinations are thought to determine the different clinical varieties of the disease It is probable that the customary diet of the present day promotes a marginal gingivitis if it is inadequately compensated for by toothbrushing this form of the disease usually responds quickly to simple hygienic treatment If treatment is deferred the cycle of changes proceeds to suppurative periodontal breakdown, which can usually be arrested by similar treatment directed to include the more deeply involved tissues Under more acute stress such as fatigue intoxication and deficiency especially of B group vitamins the ulcerative form of gingivitis may appear Outbreaks of this condition formerly considered as epidemics have occurred under group conditions especially in wartime It is also likely that a metabolic disturbance such as a transient deficiency of ascorbic acid although fully reversible elsewhere may initiate a cycle of changes in the periodontal tissues that eventuate in breakdown many years later Ascorbic acid deficiency and consequent failure of collagen formation lead to a condition in guinea pigs that resembles acute periodontal breakdown Such metabolic disturbances are particularly implicated in a form of periodontal pocket formation that is not accompanied by gingivitis

Periodontal disease has been observed or produced by nutritional hormonal or traumatic procedures in a variety of animal species (see Gupta and Shaw 1956) the findings are in general accord with the preceding discussion The observation in man of typical fusospirochetal overgrowths in periodontal exudates or scrapings is of value only in confirming the presence of a pathologic state the picture is not distinctive among the different clinical entities Treatment of periodontal disease varies with the clinical entity Chemotherapy is indicated only to relieve acute symptoms e g in ulcerative gingivitis recurrence is likely unless other measures are employed Such measures emphasize the removal of gingival and subgingival microbial pabulum and tartar and where necessary the correction of faulty dental restorations and of defects of occlusion and alignment of the teeth Local treatment of this kind supplemented by toothbrushing is frequently sufficient in itself to arrest the disease when it fails other contributory factors are sought for and, if found corrected Surgical procedures for elimination of 'pockets have been used successfully, and reattachment of severed periodontal ligament to new cementum has been accomplished both experimentally in monkeys and in man For additional details and references see Rosebury, 1952 1955 Glickman 1956, Tenenbaum et al 1957

DENTAL CARIES

Dental caries one of the most prevalent of all diseases is a distinctive process of disintegration of the hard dental tissues proceeding centripetally from the exposed surface Its unique characteristics are determined in part by the tissues in which it appears, which are either entirely noncellular (enamel) or contain only the processes of pulpal cells (dentin) and hence do not react to injury as do cellular tissues Most carious lesions begin at one of three sites in the pits and the fissures of the functional surfaces at approximating areas of contact and on surfaces of enamel or exposed cementum or dentin near the gingival margin Lesions in the first two areas are most common in the period immediately following tooth eruption i e in childhood and adolescence There is nearly universal agreement on the main lines of pathogenesis stemming principally from the studies of W D Miller in 1890 Recent research on the mechanism of the initial lesion by biochemical means (see Mandel 1955) electron microscopy (Scott and Albright 1954) and the experimental reproduction of carieslike lesions in extracted human teeth in vitro (Pigman 1955) have lent general support to this approach while amplifying it and raising many questions of detail that remain unanswered The most extensive review of the earlier literature is given in the National Research Council Survey 1952

The lesions are generally held to be initiated by acid products of bacterial fermentation of carbohydrates formed locally at sites of food accumulation or impaction Demineralization of enamel (or of dentin or cementum if the lesion begins in these tissues) appears to be the first step in the process subsequent steps may vary in sequence depending in part on the site and on the rate of progress of the lesion They include bacterial invasion pro

teolysis of enamel matrix decalcification followed by digestion of dentin or cementum and loss of tissue by minute or gross fracture consequent upon both function and forces associated with demineralization such as shrinkage and gas formation The bacterial species involved are almost certainly mixed in all instances but lactobacilli probably play a distinctive role in the mixture they alone among bacterial species recovered from caries have been found significantly increased in concentration *in relation to clinical caries activity* and their ability both to produce and to withstand low pH levels is unlikely to be coincidental The studies of Orland et al (1954) *in which germ free rats given sterilized caries* producing rations failed to develop caries confirm many previous studies in pointing to the indispensability of bacterial activity in caries Lactobacilli probably play a minor role if any in the proteolysis associated with caries The findings on all these aspects of pathogenesis are consonant with the near universality of dental caries *the apparently essential ingredients* of the process e g retentive sites on the teeth the participating micro organisms and the required pabulum as well as the susceptibility of the teeth to disintegration all appear to be nearly if not quite ubiquitous and at first glance even inescapable Yet the occasional complete absence of caries in an adult may be attributable in part to the first factor tooth form and dental architecture presumably implying a genetic component and the most probable mechanism for the protective effect of fluoride ion seems to be related to the last item *the acid solubility* of the dental tissues Nevertheless wide individual variation both in prevalence and in rate of progress of caries examined in detailed context points to the operation of additional factors and provides at least potentially means toward the practical objective of control

Experimental caries in hamsters and certain experimental lesions in rats seem to be most comparable with dental caries in man From the studies of Rosebury et al (see Rosebury 1938) Sognnaes (1948) and Gustafson et al (1953 1955) emerge a group of interdependent determinants of caries susceptibility that seem to be consistent with the human disease These include (1) the retentiveness of carbohydrate foods as determined by their physical state (2) the availability of the carbohydrate as substrate for fermentation in terms of its chemical composition (3) dental function or lack of function as it promotes or prevents food retention (4) a protective role of dietary fat perhaps acting by retarding carbohydrate solubility and (5) many other conditions less clearly defined including nutritional endocrine and other factors affecting *the structure of the dental tissues* salivary flow and composition and the pabulum retaining propensities of periodontal (interproximal and gingival) *areas* There is cogent evidence that starchy foods in dry compact form (e g raw rice or corn in animals hard fat free biscuit in both man and rats) serve as the initiating substrate for caries by forcible impaction incident to function into retentive sites *On the other hand certain sugars especially sucrose and fructose but not starch or* dextrin incorporated in the diet in dry finely divided form promote caries particularly in hamsters in proportion to their concentration In this instance it appears that impaired function evidenced after extraction of opposing teeth is necessary to permit accumulation or prevent removal of the pabulum When to such sugar diets enough distilled water was added to dissolve the carbohydrate caries in hamsters was markedly reduced suggesting that the major cariogenic effect of the diet is exerted in the mouth and not via nutritional channels (Gustafson et al 1955) Retardation of caries by inclusion of fat in the diet first noted in studies with children by Mellanby et al (see Committee 1936) has been observed in rats by Rosebury and Karshan (1935 1939) and Granados et al (1948) and in hamsters by Gustafson et al (1955) These findings suggest the possibility of preparing noncariogenic carbohydrate foods perhaps even confections by appropriate admixture with fats Among other influences on caries it may be noted that abundant evidence from *studies both in man and in experimental animals* indicates that if basic cariogenic conditions are present nutritional adequacy e g of vitamin D decreases or retards the progress of the lesion

Diagnosis of dental caries is made clinically with the aid of roentgenograms Counts of the concentration of lactobacilli in saliva and other caries activity tests are useful principally in confirming a clinical diagnosis of rampant caries and as a sensitive index in the individual patient of the progress of attempts at dietary control Control of unusually high

caries activity in children has been effective by drastic limitation of carbohydrate intake The use of a variety of dentifrices containing alleged anticariogenic agents is of doubtful utility but carefully supervised toothbrushing with any dentifrice or none where it can be practiced may have some value Caries can be arrested by the traditional dental practice of removing involved tissue and replacing it with inert filling materials The fluoridation of public water supplies to the average level of 1 part of fluoride per million parts of water has been fully established as nontoxic industrially innocuous and effective in reducing the community prevalence of caries by 60 per cent or more most actively in children born and reared on fluoridated water but with distinct value when started at any age (see Arnold 1957 Hayes Littleton and White 1957) This now widespread practice is probably the most thoroughly studied of all public health procedures its effectiveness is beyond serious question but it is capable of solving only approximately half the problem of dental caries The effectiveness of topical application of 2 per cent NaF to the teeth of children has also been clearly demonstrated and is applicable in rural areas where community fluoridation is not feasible The mechanism of inhibition of caries by fluoride seems to involve its incorporation in enamel as fluorapatite either preeruptively or by ion exchange in later life

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32

Principles of Sterilization

GENERAL

Asepsis refers to the use of technics that avoid the introduction of viable microbes and *sterilization* to procedures that employ a physical or a chemical agent to render a material free of such organisms The process of sterilization of an object by means of a chemical agent is generally referred to as *disinfection* while the topical application of chemicals to a body surface for the same purpose (or more precisely to eliminate all pathogens) is referred to as *antisepsis* The same compounds are often used as disinfectants and as antiseptics As will be discussed in greater detail below these compounds are toxic to all kinds of cells and hence cannot be employed in systemic therapy of infection *Chemotherapy* in contrast depends on a highly selective cytotoxicity chemotherapeutic agents are used to antagonize parasites within the host tissues at concentrations that the host can tolerate

Antiseptics are widely used for sterilization of the skin in preparation for surgery but in prophylactic application to wounds or in the topical therapy of superficial infections antiseptics have been largely replaced by chemotherapeutic agents The latter are less damaging to the tissues and also more likely to remain active after diffusion into the superficial layers of the treated surface

Antibacterial effects are divided into *bacteriostatic* and *bactericidal* actions (note spelling) The former denotes a reversible inhibition of proliferation the latter an irreversible "killing" or destruction of viability Both actions are important in chemotherapy but bactericidal action is required for disinfection

CRITERIA OF VIABILITY

The fundamental criterion for the viability of a microbe is simpler than the criteria used for differentiated higher organisms it is the ability to propagate indefinitely when placed in a suitable environment Hence the individual cell which has become nonviable according to this criterion is most precisely described as sterile rather than dead or "killed" Depending on the means used sterilization may or may not be associated with changes in morphology (e g lysis) staining properties motility and respiration or other enzymatic activities Thus a boiled suspension of vegetative cells will show no respiration or other vital activity whereas a suspension treated with ultraviolet or x rays will not only exhibit a variety of metabolic activities but will even reveal when observed microscopically after plating that many of the nonviable cells undergo several divisions before proliferation ceases Similarly after irradiation spores can often germinate, but they exhibit little or no division It follows that indirect biochemical or microscopic criteria for measuring bactericidal action cannot be considered reliable unless checked by direct tests for viability

Another difficulty in estimating bactericidal action precisely arises from the fact that two different media both adequate for ordinary cells of a given species might give quite different viable counts when inoculated with damaged cells Such an effect is readily seen

for example following ultraviolet irradiation (see below) An extreme case involves mercuric ion whose antibacterial action depends on combination with sulfhydryl groups in the cell As early as 1889 it was found that Koch had overestimated the disinfectant action of $HgCl_2$ for when anthrax spores had been sterilized by exposure to the compound for a week they could be resurrected by washing with a solution of H_2S which forms a highly insoluble compound with Hg^{++} and hence reverses its combinations in the cell Fildes has shown that the process of reversal can be carried still further by cultivation in media containing sulfhydryl compounds such as cysteine or thioglycollic acid And in animals administration of an appropriate sulfhydryl compound (BAL) has been found to permit infection by mercury treated pathogenic organisms which were harmless to untreated animals

Perhaps it is better not to attempt to define sterility or death in absolute terms but rather to define it in operational terms subject to changes with the development of new technics A material is sterile if it is impossible by available technics to demonstrate viable bacteria in it The considerations introduced here assume practical importance in connection with the preparation of vaccines which are often sterilized as gently as possible in order to retain antigenicity It is important to remember that for this purpose artificial culture media do not have the final word as a practical matter pathogenic bacteria (and viruses and other microbes) have been adequately sterilized if they are unable to propagate in the animal body

It should be emphasized that *sterilization* is not identical with *destruction* of bacteria or their products though the terms are often loosely interchanged For example in preparing solutions for intravenous administration it is not sufficient to take pains to ensure sterility it is also necessary to avoid introduction of pyrogens (fever producing substances) through previous bacterial contamination since these products remain active despite subsequent autoclaving or filtration Hence in the preparation of biologics and parenteral fluids the distilled water the reagents and the glassware used must satisfy criteria of purity quite different from those required for analytical chemical work

History

In the perspective of civilization it is only recently that the role of micro organisms in putrefaction and decay was discovered but practical means of preventing these processes are among the early household arts Perishable foods have long been preserved by drying by salting and by fermentations that yielded a high concentration of lactic acid (soured milk cheese sauerkraut ensilage) The embalming practiced by the ancient Egyptians may be regarded similarly but its success may have owed more to the dry climate of the Nile valley than to the essential oils used In more modern times Appert introduced the art of canning 50 years before Pasteur s researches gave it a rational basis

Disinfection of pure cultures of bacteria was first studied in 1871 by Koch employing $HgCl_2$ and in 1897 Kronig and Paul obtained quantitative evidence for the gradual nature of the process However the field remains largely empirical and while it is of practical and commercial importance fundamental advances have been limited Most of the recent interest in antibacterial agents has naturally been concerned with those that are useful in chemotherapy these will be considered in the next chapter

Differential Susceptibility

The various bacterial groups differ widely in their sensitivity to heat and to various disinfectants thought not to radiation they range from the pneumococcus which tends to autolyze in all but the most favorable environments to the tubercle bacillus which resists strong acid and alkali However the reactions may be quite specific the tubercle bacillus is much more susceptible than most bacteria to fatty acids (soaps) Within a given genus the species behave quite similarly While generalizations are frequently made concerning the behavior of the classes of bacteria particularly the difference in the susceptibility of gram positive and gram negative bacteria to various disinfectants extensive testing generally reveals striking exceptions The differences in susceptibility are only relative the more resistant organisms requiring a more intense or prolonged exposure to the sterilizing agent It must also be remembered that the process of sterilization is gradual The expo

sure of sputum to an appropriate concentration of alkali for example may sterilize all the cells of most bacterial species present while the tubercle bacilli and some staphylococci and streptococci are more resistant Yet, the vast majority of the tubercle bacilli them selves may be sterilized, the successful diag nostic cultivation depending on the viable residue

The susceptibility of the cells of a given species varies markedly with their physiologic state The cells in a young culture which are larger and more rapidly growing than those in an old culture approaching nutritive exhaus tion are also more susceptible to various physical and chemical agents There may well be a further difference in the susceptibility of the cells at different ages between birth and fission Much more important than these effects from a practical point of view, is the difference between vegetative cells and the spores formed by some species spores are much more resistant to abnormal temperatures and to chemical agents Therefore they seem to have the biologic function of permitting sur vival through hard times The reasons for the increased resistance of spores are poorly un derstood They are said to have thicker cell walls than vegetative cells but while these could hinder the penetration of chemicals they could not be responsible for the increased re sistance to heat Spores also have a lower water content which may hinder protein de naturation As a practical matter sterilization *procedures must be adequate for the spores* which may be present the vegetative forms are then taken care of automatically

The usual method of measuring disinfectant action consists in mixing a given inoculum with a given concentration of disinfectant and at successive intervals transferring a loopful or other small sample to a large volume of broth or to a solid medium the presence or the number of viable survivors is recorded The rate of sterilization depends on a large number of variables nature and concentration of the disinfectant strain and physiologic state of the bacteria number and population density of the bacteria temperature pH and concen tration of salts proteins and other protective or harmful materials Since data are available for only a small and arbitrary fraction of the possible permutations of these variables the action of the various agents will be discussed in a general way with little quantitative detail

PHYSICAL AGENTS

Moist Heat

This is the method of choice for sterilizing all materials except those that would be dam aged by it The process is rapid, all organisms are susceptible and the vapor penetrates clumps and reaches surfaces that might be missed by a chemical disinfectant Fungi viruses and the vegetative cells of all patho genic bacteria are sterilized within a few min utes at from 50° to 70° C and even the much more resistant spores of the anthrax bacillus and of other pathogens are sterilized within a few minutes at 100 C In conse quence syringes, needles and instruments for minor surgery can be sterilized adequately by boiling for from 10 to 15 minutes in water, or even better in a dilute solution of alkali (e g washing soda)

However there are thermophilic sapro phytes which have the remarkable property of surviving and even multiplying rapidly at temperatures as high as 80° C their spores can survive boiling for 24 hours For major surgery and bacteriologic cultivation absolute sterility is essential and so even the most re sistant spores must be sterilized For this purpose Pasteur introduced the use of steam under pressure the autoclave has since be come the symbol of the bacteriologic labora tory *In order to ensure sterilization* of the most resistant spores that may be present it has become customary to expose material to steam at a temperature of 120 C for 20 to 30 minutes This temperature is attained by steam at a pressure of 15 pounds (in excess of atmospheric pressure) at altitudes near sea level at high altitudes slightly higher pres sures are necessary

In using an autoclave it is important that flowing steam be allowed to displace the air before building up pressure for steam mixed with air (superheated steam) fails to pene trate porous material as rapidly as saturated steam and it heats up objects much more slowly since the steam cannot condense on sur faces unless saturated The condensation of steam with a heat transfer of 540 cal /Gm permits even large objects to reach the ambient

temperature within a few minutes It is also important that vessels be loosely plugged or capped and not completely filled with liquid in order to permit free ebullition of the air contained Modern autoclaves are provided with a steam jacket which makes it possible to keep the contents of the autoclave hot after replacing the steam with air in order to dry off the condensed water rapidly This is essential for large cloth or cotton objects since these materials are effective bacterial filters only when dry

Some media contain components which would be rapidly destroyed at 120 C but can withstand 100 C These media are sterilized by exposure to flowing steam at atmospheric pressure in an Arnold sterilizer on 3 successive days The theory underlying this *fractional sterilization* (Tyndallisation) is that vegetative cells will be destroyed at 100° C and any spores present will germinate during the intervals of storage The procedure is obviously applicable only to nutrient media which will promote germination A modification is the inspissation at 85 C of egg media for tubercle bacilli Fractional sterilization was widely used for the complex older media but is much less common today

The process of *pasteurization* was introduced by Pasteur to prevent the acidification of wine by bacterial oxidation following the alcoholic fermentation by yeast However this application has remained limited since to the French winemakers increased shelf life could not make up for real or fancied loss of bouquet Meanwhile the process has developed great importance for public health practice as a means of preventing milk borne infection Its effectiveness depends upon the fact that the common milk borne pathogens (tubercle bacillus salmonella streptococcus and brucella) do not form spores hence they are sterilized by a moderate degree of heating whose effect on flavor is tolerable to all except a few connoisseurs

Pasteurization consists of heating at 62 C for 30 minutes or at a slightly higher temperature for a briefer period A useful check for pasteurization arises from the fact that phosphatase a readily measured enzyme is always present in raw milk and is destroyed by this amount of heating

Dry Heat

Weak intramolecular and intermolecular bonds (e g hydrogen bonds) in proteins and other macromolecules are much more labile in the presence of water than in its absence (Homely evidence is provided by the usefulness of steam in pressing fabrics) Since the denaturation of proteins depends upon the rupture of such bonds it is hardly surprising that bacteria like enzymes are much more stable in the dry state Indeed vegetative typhoid bacilli thoroughly dry and sealed in a vacuum are said to survive 30 minutes at 115 C Ordinary air-dried organisms are somewhat more susceptible but not as much as when surrounded by water or steam In consequence sterilization by dry heat requires 160° C for 1 to 1½ hours which ruins most fabrics and somewhat weakens cotton plugs Dry heat has the further disadvantage that hot air penetrates porous materials much more slowly than steam so that after an hour in an oven at 160 C the center of a large package of surgical dressings may not even have reached 100 C Sterilization by dry heat is ordinarily used only for glassware and metal objects Intense dry heat is used in the process of flaming which is indispensable in the bacteriologic laboratory and in disposing of infectious materials by incineration

It is often desirable to sterilize within a few minutes instruments unexpectedly required in the course of a surgical operation This may be accomplished by high pressure autoclaving (moist heat) or immersion in hot oil (dry heat) it is necessary to use oil with a high flash point

Mechanism of Sterilization by Heat

Heat sterilization of bacteria was shown by Chick in 1910 to parallel in several respects heat denaturation of proteins Both processes (1) have an exponential time action curve (which will be discussed in detail later) (2) have a temperature coefficient of several hundred (Q_{10} = the ratio of the rate at a given temperature to the rate at a temperature 10 C lower) in contrast with the Q_{10} of 2 to 3 observed for most chemical reactions and (3) occur in approximately the same range of temperature Even the anomalous resistance of thermophils and spores has some

parallel in protein chemistry since some enzymes (e g myokinase ribonuclease) are not inactivated by boiling under certain conditions Bacteria and proteins further resemble each other in showing some protection by neutral substances (such as glucose or glycerol) added in relatively high concentrations to the surrounding medium and in being even more resistant to heat when they are dry It is concluded that the site of thermal damage in bacteria is probably their proteins which include of course their enzymes

The heat sensitivity of a bacterial species is a useful characteristic to measure for taxonomic and other purposes and since the rate of sterilization can be determined with fair precision and often follows an exponential curve for much of its course this sensitivity is probably best defined in terms of the half life under a given set of conditions However most studies of sterilization have been concerned with practical considerations and hence have chosen complete sterilization as the end point The thermal death time is that necessary for complete sterilization at a given temperature while the 'thermal death point is the temperature necessary for complete sterilization after a given period (e g 10 minutes)

Efforts to develop relatively heat resistant strains of bacteria by repeated subinoculation of the survivors of heating have failed to produce a really significant effect in contrast with the successful development of drug resistance in analogous experiments

Cold

Most bacteria are resistant to cold and will survive much longer in the refrigerator than in the incubator Freezing kills approximately 50 per cent of a suspension of *E coli* but this is undoubtedly a mechanical effect rather than an effect of cold since repeated freezing and thawing is much more destructive than maintenance of the frozen culture Further cooling to temperatures as low as that of liquid air (—186° C) may cause no harm A useful method of indefinitely preserving strains of bacteria viruses and even cells as complicated as spirochetes or plasmodia is storage in sealed ampoules surrounded by CO ice (—78 C) At temperatures of —5 to —25 C such as are provided by mechanical freezers bacteria do not remain viable indefinitely since these temperatures exceed the eutectic point of the salts present and therefore permit chemical changes in the tiny pockets of saturated salt solution which are present

Desiccation

Though the number of viable bacteria in a droplet may be reduced considerably by drying a bacterial cell that survive the process of drying may then retain its viability in a thoroughly dried state for years even without refrigeration The initial loss of viability is minimized by carrying out the drying procedure from the frozen state (lyophilization) in the presence of a protective protein solution this procedure is widely used for the preservation of cultures Lyophilization is used similarly to preserve antisera and other proteins The increased stability of a cell in the dried state observed over a long period of time at moderate or low temperatures parallels the already noted difference in effectiveness of moist and dry heat as rapid sterilizing agents at high temperatures Both processes undoubtedly reflect the usual participation of water in protein denaturation

Ultraviolet Radiation

The spectrum of visible light ranges from a wave length of 7 500 Å (red) to 4 000 Å (violet) 1 Å (Ångstrom unit) being 10^{-8} cm or 0 1 millimicron (mμ) The biologically active part of the ultraviolet spectrum extends from 4 000 Å down to 2,000 Å

The well known sterilizing effect of sunlight on bacteria is due mainly to its content of ultraviolet light By far the greatest proportion of the ultraviolet radiation reaching the earth s atmosphere from the sun is screened out by the ozone present in the outer regions of the atmosphere otherwise most species present on the earth s surface could not survive The lower limit of wave length of sun light reaching the earth s surface in quantity is approximately 2 900 Å Practically all the ultraviolet light of sunlight is screened out by ordinary glass as is shown by the failure of such light to produce sunburn While no sharp upper limit can be placed to the bactericidal wave length of light appreciably rapid sterilization is first shown at 3 300 Å and increases rapidly with decrease in wave length Intense ultraviolet light of predominantly shorter wave

lengths, 2 400 to 2 800 Å which are much more bactericidal per unit of radiant energy than the ultraviolet components of sunlight can be produced by mercury vapor lamps In experiments involving such radiation it is necessary to expose the bacteria either in an uncovered vessel or in one made of fused quartz (silica) which does not absorb light of these wave lengths (Cf Hollaender 1942)

Sterilization by ultraviolet light is subject to the laws of photochemistry (1) The mere passage of light through a transparent medium is without chemical effect only absorbed radiant energy can be effective (2) Light is absorbed in minimal units of energy called quanta The energy of a quantum is inversely proportional to its wave length a molecule which has absorbed a quantum of ultraviolet light has consequently received a greater increment of energy which might activate a chemical reaction than a molecule which has absorbed a quantum of visible light (3) The number of quanta absorbed is proportional to the product of the intensity and the duration of the radiation as well as to the absorption coefficient of the irradiated material There is consequently no basis for the belief that bacterial vaccines sterilized by very brief exposure to intense radiation are less damaged as antigens than those sterilized by a comparable amount of radiation spread over a longer period of time Ultraviolet absorption by cells is due chiefly to nucleic acids in which the purines and the pyrimidines absorb heavily with a maximum at 2 600 Å and to proteins in which the aromatic rings of tryptophan tyrosine and phenylalanine absorb moderately with a maximum at 2 800 Å The sterilization action spectrum (i e the efficiency of sterilization by radiation of various wave lengths) parallels the absorption spectrum of the bacteria indicating that absorption by either nucleic acid or protein can have a lethal effect

The energy of the absorbed quantum which activates molecules by producing increased interatomic vibration or else excitation of electrons to a higher energy level may follow a variety of paths The activated molecule may rupture any of a variety of chemical bonds and form new bonds with adjacent molecules or it may transfer most of its extra energy by collision to an adjacent molecule which then undergoes chemical reaction or the energy may be entirely dissipated by collision as increased translational energy (heat) without any chemical change

Studies of irradiated solutions have shown depolymerization of nucleic acids and both disaggregation and aggregation of proteins which are included in the vague term denaturation

Ultraviolet radiation causes formation of ozone (O_3) in air and of hydrogen peroxide (H_2O_2) in water containing dissolved oxygen For this reason intensely irradiated media are transiently toxic to subsequently inoculated bacteria

The quantum yield of ultraviolet sterilization is small the average *E coli* cell has absorbed over 10^6 quanta by the time it is sterilized and even the smallest viruses which are killed by a single radiation induced ionization (discussed later) require many ultraviolet quanta This means that the vast majority of the absorbed quanta are dissipated without chemical reaction or that most of the molecules or chemical groups affected are not essential for survival undoubtedly both factors are involved

Ultraviolet irradiation has been shown to produce mutations in micro-organisms as well as in the germ cells of higher animal The concept of the sterilizing effect as a lethal mutation due to alteration of the ultraviolet absorbing nucleoproteins of chromosomes or of their analogues in bacteria (Lea 1947) will be discussed below (p 661) While such a process very likely does occur there is much recent evidence that the bactericidal action of ultraviolet irradiation is due to other effects in addition to a direct one on genetic material (1) The time action curve of sterilization often deviates markedly from the exponential shape discussed on page 658 (2) mutations and sterilization can be produced by chemicals (e g hydrogen peroxide) which are known to be produced in the medium by irradiation (Wyss Stone) (3) certain strains of *E coli* which are especially susceptible to ultraviolet sterilization give rise to radiation resistant mutants (e g strain B/r) that are much more resistant to such sterilization (Witkin) (4) a large fraction of the lethal effect of ultraviolet irradiation can be reversed by exposure before incubation to intense irradiation of visible wave lengths (Kelner) and (5) a greater lethal effect is exhibited on incubation of the irradiated cells in a simple medium compared with a rich medium presumably some spontaneous reversal of the lethal effect occurs during the longer lag

phase of the bacteria on the simpler medium (Roberts)

Inexpensive mercury vapor lamps are now commercially available as sources of ultra violet radiation They have been used in the preparation of bacterial and viral vaccines there being some evidence that sterilization by this means causes less destruction of de sirable antigens than the use of heat or chemi cal agents In preparing such vaccines it is important in order to avoid occasional sur vival of pathogenic bacteria that the suspen sions be free of clumps be stirred sufficiently to ensure uniform exposure and be subjected to enough radiation to provide a generous margin of safety

Ultraviolet lamps have begun to find use in preventing air borne infection in public places and surgical operating rooms they have been shown to decrease cross infection in hospital wards and in animal colonies

Ultraviolet lamps are similarly useful in the laboratory for decreasing contamination of cultures and infection of workers In bacterio logic transfers however it is undesirable to expose loops or open plates containing bacteria to direct irradiation though bacteria protected by glass may be exposed briefly It is also important to protect the eyes of workers since even brief exposure of the cornea causes severe irritation with a latent period of 12 hours

Tuberculous cross infection in rabbits, for example caused by dust from bedding con taminated by urine can be completely pre vented by ultraviolet lamps it is not neces sary that a wall of ultraviolet light be formed between the cages but is sufficient if the air is exposed to enough radiation in the course of circulating through the room (Lurie 1945) However surfaces are sterilized only by direct exposure Although the practice of attaching lamps to public toilet seats appears to be based more on commercial enterprise than on hygienic principles ultraviolet lamps do have real epidemiologic value and may find in creasing use in the future The patients who have become accustomed to the germicidal smell of phenol and essential oils in medical establishments probably will be educated to enjoy the faint but more significant odor of ozone

Photodynamic Sensitization (Blum, 1941)

Certain dyes, such as methylene blue, rose bengal and eosin sterilize bacteria and viruses in strong visible light at concentrations of dye much lower than those required in the dark. Under similar circumstances they hemolyze red blood cells and denature proteins, includ ing toxins and antibodies The effect is pro duced by a comparatively small number of dyes which vary in structure but have in common the property of fluorescence Photo sensitization has not been of much practical value in bacteriology but must be borne in mind when working with potentially photosen sitizing dyes

These dyes in contrast with nonfluorescent dyes, retain an absorbed quantum for a com paratively long time (10^{-6} to 10^{-8} sec) and then release a large fraction of its energy in a single unit either by fluorescent radiation or by transfer by collision with another molecule, the latter may then undergo chemical change Since free oxygen is required for photodynamic reactions (in contrast with reactions produced by ultraviolet radiation) it appears that the dye activates either an oxygen molecule or a substrate molecule and thereby brings about the oxidation of otherwise stable substances

Even in the absence of dyes visible light when intense, is capable of killing bacteria presumably via physiologically occurring pho tosensitizing substances such as riboflavin and porphyrins This effect makes it inadvis able to expose bacterial cultures to direct sun light even when protected by glass BCG vaccine in glass ampules for example, has been observed to lose all viability and effec tiveness on exposure to sunlight as may easily occur in field stations in tropical countries

X Rays and Other Ionizing Radiations

X rays and the γ rays of radioactive ele ments are electromagnetic radiations of wave length 0 01 to 10 Å Other ionizing irradia tions include beams of high speed electrons (cathode rays and the β rays emitted by radioactive substances) of high speed helium nuclei (α particles emitted by radioactive sub stances), and of high energy protons neu trons or other particles produced by various accelerating devices These irradiations are lethal in sufficient dosage to all cells includ ing bacteria the differential sensitivity of

various cell types allows ionizing irradiations to be beneficial in the treatment of malignancy X rays are also used clinically in the treatment of chronic bacterial and fungus infections but since the doses used are too low to effect much sterilization any beneficial effect produced probably depends upon a tissue response

Ionizing radiations are not of much practical use in bacteriology though work is in progress on the sterilization of materials (e g packaged foods) without heat through the use of electron beams However bacteria are widely used in experiments aimed at analyzing the lethal and the mutagenic (mutation producing) action of ionizing radiations The quantum energy values of all these radiations are hundreds to thousands of time as great as those of ultraviolet light consequently their mode of action is entirely different Instead of having each quantum absorbed by a molecule of appropriate configuration a part of the energy of a quantum is contributed to each of several hundred atoms of any kind which lie in its path regardless of the chemical structures in which those atoms are involved The absorbing atoms are ionized by the ejection of an electron which is the reason for the collective term ionizing radiations The molecules containing the ionized atoms then undergo chemical change involving the rupture and the formation of bonds The ultimate results are much the same as those caused by ultraviolet radiation except that (1) they are chemically more varied (2) the energies involved are so high that there are no duds and (3) a single quantum alters many molecules in its path In consequence a single quantum may inactivate more than one bacterium The biologic action of these radiations has been reviewed by Lea (1947)

It has been shown recently (Hollaender 1951) that the presence or the absence of oxygen affects susceptibility of bacteria to ionizing irradiations Therefore it appears that these radiations like ultraviolet radiation exert indirect as well as direct lethal effects

Ionizing irradiations produce in addition to their lethal effect a type of damage that affects cell division more than it affects growth in consequence on first cultivation very elongated bacteria appear In contrast with the all or none lethal effect of radiation this effect is graded in proportion to the dose to which the cell has been exposed It is not known whether the effect is due to accumulation of poisons or destruction of metabolites in either case metabolic processes eventually compensate for the change Similar effects are produced by many other inhibitory agents including some chemotherapeutics

Ultrasonics

Sound waves which are longitudinal mechanical vibrations are of course quite different in effect from the transverse electromagnetic vibrations of light In the range exceeding the audible (called supersonic or ultrasonic) with a frequency of 15 000 to several hundred thousand per second they coagulate protein solutions disperse a variety of materials and sterilize and disintegrate bacteria Even audible sonic waves in sufficient intensity have been reported to be weakly bactericidal The effect has not been of practical value as a means of sterilization but has been found useful in extracting enzymes and antigens

Filtration

It is possible to obtain bacteria free filtrates by the use of filters with a maximum pore size of 1 5 μ or less The Seitz filter of asbestos and the Berkefeld filter of diatomaceous earth are still widely used but are quite adsorptive in addition the Seitz filter contributes considerable Ca and Fe to the solution and therefore causes clotting of citrated plasma The Chamberland unglazed porcelain filter has been modified with more accurate control of pore size by the modern porcelain industry and effective sterilizing filters of sintered (fritted) glass (Corning UF) are also available These two types of filters adsorb very little material and are ideal for the sterilization of sera media which cannot be heated (e g media containing soluble proteins) and experimental materials in which possible effects of heating are to be avoided They also permit quantitative recovery of bacteria for chemical or other purposes These advantages also apply to the thin plastic films of accurately graded porosity that have become available recently (e g Millipore filters) and these filter considerably faster than porcelain or glass The availability of such filters facilitates separate analysis of the changes that take place in the bacteria and in the medium during cultivation

CHEMICAL AGENTS

General

The action of disinfectants on a bacterial population is not instantaneous but is cumulative with time, and the rate increases with concentration and temperature, as is true of practically all chemical reactions In contrast with the action of most of the bactericidal chemotherapeutic agents the attack of disinfectants does not require metabolic activity on the part of the victim In terms of their mechanism of action disinfectants appear to fall into two classes those that cause lysis by dissolving lipids from the cell membrane, and those that cause denaturation of macromolecules This denaturing effect is demonstrated most readily with proteins but in some cases it probably involves desoxyribonucleic acid or ribonucleic acid

Disinfectants are classified in pharmacology as protoplasmic poisons a vague term describing substances which depress the activity of all sorts of cells This widespread effect implies a rather nonspecific affinity for large classes of cellular constituents therefore it is not surprising that bacteria refuse to develop much resistance to these agents (In contrast the chemotherapeutics have a much more selective action and bacterial strains frequently become resistant to them)

It may be noted that whether a given substance is a meat or a poison to bacteria is often simply a matter of concentration oxygen salts fatty acids some vitamins and amino acids and glycerol in high enough concentrations may be bacteriostatic and even actively bactericidal to bacteria that utilize or require them for growth

Those antibacterial agents that are capable of ionizing as acids (anionic surface active agents including phenols dyes and organic acids including acidic chemotherapeutic agents) are more active with increasing acidity of the solution while the opposite is true of cationic reagents This effect of pH can be partly ascribed to an increased affinity of bacteria for an ionized disinfectant due to increasing ionization (in the opposite direction) of bacterial proteins In addition the favorable pH effect decreases the ionization of the disinfectant and it is a well established rule of cellular physiology that many ions penetrate cell membranes less readily than the corresponding un ionized molecules Alternatively the phenomenon can also be analyzed in terms of competition between the drug and H^+ or OH^- ions (Albert, 1951)

Those disinfectants for which homologous series are available with varying hydrocarbon chains (e g , alcohols, surface active agents) show an increase in bactericidal potency with increasing chain length and there is a parallel increase in surface tension depression Both effects show a maximum which varies from 8 to 10 carbon atoms for the alcohols and substituted phenols to 12 to 18 for the more soluble detergents

The increasing size of the hydrophobic portion of the molecule tends to drive it out of aqueous solution, either to form an oriented layer at an air water interface (surface tension effect) or to be adsorbed on the surface of a bacterial cell membrane or macromolecule The decrease in antibacterial activity and in surface tension effect at chain lengths above the maximum is due to insufficient solubility This relative insolubility is often inapparent and takes the special form of an increased tendency to aggregate in micelles which form colloidal solutions and are less surface active than the individual molecules Disinfectants thus obey Traube's rule which correlates the efficacy of narcotics and certain other pharmacologic agents with their effect upon surface tension

Distilled Water

Distilled water is a less satisfactory menstruum than broth or other complex media for the preservation of bacteria although with many species it may be used as a temporary diluent without causing any loss of viability The agents of water borne infections such as the typhoid bacillus have been reported to survive for months in tap water In general bacteria differ strikingly from tissue cells in being quite indifferent to osmotic effects over a wide range However a few species are lysed by distilled water a phenomenon referred to as plasmolysis In some cases the apparent toxic action of water may be due to traces of heavy metal ions whose toxicity would be reduced in a diluent that is rich in organic materials (such as broth) or even in a solution of buffer salts

Acids and Alkalis

The accumulation of lactic acetic and other organic acids limits the density of growth of many microbes the yield and the reproducibility of certain economically useful fermentations and the viability of stationary phase cells Attempts to control these phenomena were largely responsible for initiating the early studies that led to the understanding of acid base potentials and particularly of the distinction between titrable acid and H^+ concentration

The growth of most bacteria is restricted to a pH range of about 5 to 9 and for some species the range is even narrower However sulfur oxidizing bacteria can accumulate sulfuric acid up to a concentration of several per cent Yeasts and molds generally thrive at pH values too low for most bacteria and this property is useful in selective cultivation

Solutions that are sufficiently acid or alkaline are actively bactericidal For example HCl or H_2SO_4 in a concentration of 0 01 N completely sterilizes a suspension of *E coli* in less than 40 minutes However mycobacteria are somewhat more resistant to acid and alkali it being common practice to liquefy sputum by exposure for 30 minutes to approximately 1 N NaOH or H_2SO_4 at 37 C Gram positive staphylococci and streptococci frequently survive this treatment too It should be pointed out that tubercle bacilli are by no means completely resistant to such treatment its success in their isolation depends rather on the survival of a fraction of the population

Weak organic acids exert a greater effect than can be accounted for by the pH this effect probably depends upon the greater permeability of cell membranes for un ionized molecules

Salt

The practice of inhibiting bacterial growth by pickling in brine or by treatment with solid NaCl has been used for many centuries as a means of preserving perishable meats and fish There is considerable variation in bacterial susceptibility NaCl prevents the growth of tubercle bacilli at a concentration of 2 per cent *E coli* at around 6 per cent and *B subtilis* only at over 9 per cent In contrast certain organisms found in the Dead Sea in brine etc require 10 to 12 per cent NaCl for growth and are therefore called halophilic

Physiologic saline is not very suitable as a diluent for bacteria though it is widely used because of its inevitable presence in laboratories where serologic tests are performed Flexner observed that a trace of Ca^{++} prevented the disintegration of meningococci in this diluent therefore a balanced salt solution appears to be desirable for some bacteria just as Ringer long ago observed for heart muscle

Metallic Ions

Mercury and silver form the most antibacterial salts $HgCl_2$ or $AgNO_3$ prevent growth of many bacteria in concentrations of less than 1 part per million and sterilize small inocula in somewhat higher concentrations The various metallic ions can be arranged in a series of decreasing antibacterial activity roughly the same for a variety of bacteria with these two at the head of the list then the other heavy metals the iron group the alkaline earths and finally the alkali metals of which the action of Na^+ has been described Pharmacologists coined the term oligodynamic action to refer to the extremely high effective dilutions of metallic ions the assumption being that the presence of comparatively few ions exerted a remarkable effect on the cell The effect is due however to the great affinity of certain proteins for these ions which causes bacteria to take up relatively large amounts from very dilute solutions It has been found that bacteria trypanosomes or yeast killed by Ag^+ contain 10^5 to 10 Ag^+ ions per cell which is the same order of magnitude as the estimated number of enzyme protein molecules per cell (Clark 1937) Because of this uptake the bactericidal concentration is markedly affected by the inoculum size and the presence of proteins in the medium

It was mentioned earlier that the antibacterial action of Hg^{++} could be readily reversed by sulfhydryl compounds whose affinity for Hg^{++} long ago gave rise to the term mercaptan There is good reason to believe that the biologic effect of this metal as well as of arsenicals depends upon similar combinations with sulfhydryl groups within the cell The reversibility of the apparent bactericidal effect is readily understandable since a large number of enzymes have been shown to

be reversibly inactivated by oxidation or other alteration (including combination with Hg^{++}) of their sulfhydryl groups However, the action of heavy metals on bacteria is quite complicated since they undoubtedly combine with a variety of cellular constituents and eventually cause irreversible damage

Mercuric chloride was long a popular dis infectant in 1 5 000 solution though it is being largely replaced by organic surface active compounds which are more rapid and irreversible in action less expensive and not so poisonous to man The chemotherapeutic use of mercury or its salts in the treatment of syphilis is of historic interest only A va riety of organic mercury compounds in which one of the valences of Hg is free to combine with protein or else the Hg is united to the compound via a labile S or N linkage which permits slow dissociation of mercuric ion are used as relatively nonirritating antiseptics for skin and mucous membranes and as antibac terial preservatives (e g merthiolate mer curochrome metaphen)

Silver has long been used as a relatively in effective antiseptic in the form of a proteinate which slowly releases silver ions Organic com pounds of arsenic bismuth and antimony have been used in the chemotherapy of syphilis and certain protozoal diseases Gold has also been used chemotherapeutically but with question able value Copper salts have had tremendous importance as fungicides in agriculture but not in medicine

It might be noted that most metallic sur faces become coated with oxides which exert an antibacterial effect through contributing ions to a contiguous solution Therefore the old tin cup may have been less of an epi demiologic hazard than the common drinking glass

Inorganic Anions

Inorganic anions are much less toxic than some of the cations For some reason boric acid has found wide use as an extremely mild antiseptic Fluoride which inhibits several en zymes is toxic to many bacteria but in con *centrations* far above those noted for Hg Potassium tellurite which is particularly in hibitory to gram negative organisms and least to corynebacteria is used in the selective cul tivation of diphtheria bacilli

Halogens

Tincture of iodine (a 7% or 3 5% solution in alcohol containing KI) is one of the most rapidly acting bactericides Although its pain ful and destructive effect on exposed tissue has led it to be discarded for the treatment of large wounds, it is an excellent antiseptic for skin and minor wounds It can readily be shown to combine irreversibly with proteins

Chlorine combines with water to form hypochlorous acid (HOCl) an oxidizing agent which is rapidly bactericidal Dakin s solution freshly prepared hypochlorite of a standard strength, was widely used in World War I to irrigate wounds It was largely replaced by the azochloramides (e g , Chloramine T), or ganic compounds with a labile Cl atom attached to N which release free chlorine in solution and are less irritating to tissues Both compounds have now been replaced by modern chemotherapeutic agents in the prophylaxis and the treatment of wound infection Chlorine in a dilution of a few parts per million is widely used to disinfect drinking water and swimming pools

Oxidizing Agents

Hydrogen peroxide (H_2O_2) which is mar keted as an antiseptic in a 3 per cent solution cannot be strongly recommended even though patients derive satisfaction from seeing the *bubbling* that occurs when it comes into con tact with catalase in the tissues Bacteria vary widely in their susceptibility since some bac teria possess catalase anaerobes in general lack this enzyme Potassium permanganate ($KMnO_4$) is of value as a urethral antiseptic in concentrations around 1/1 000 or less These as well as the halogens presumably act by oxidizing and thereby inactivating en zymes (Knox et al 1948)

Formaldehyde and Ethylene Oxide

Formaldehyde (HCHO) is marketed as a 37 per cent aqueous solution (formalin) It is particularly valuable as a means of sterilizing bacteria and inactivating bacterial toxins with out destroying the antigenicity that is essential for their use in immunization For this purpose exposure to a 1 1 000 solution for a number of hours is usual It is also used for urinary antisepsis by oral administration of a com

pound (methenamine) which releases formaldehyde in acid urine Formaldehyde is capable of reacting with a variety of groups especially NH and OH groups which are abundant in proteins and nucleic acids By replacing a hydrogen atom on two such groups on separate molecules it forms methylene bridges which link the molecules permanently and it thereby hardens or fixes the tissue and inactivates most enzymes However there are enzymes (e g verdoperoxidase) which resist inactivation by formaldehyde and therefore can be demonstrated histochemically on tissues fixed by formalin Such a consideration helps us to understand the remarkable diversity of microbial life which includes even molds capable of growing in formalin

Formaldehyde is sometimes used as a gas for sterilizing dry surfaces Ethylene oxide has recently been introduced for the same purpose and is widely used in industry Its mechanism of action is similar to that of formaldehyde

Phenols

Since Lister started spraying phenol (carbolic acid) through his surgical operating rooms this compound has been considered the standard disinfectant Actually it is one of the least active compounds mentioned in this chapter and must be used in a concentration of several per cent Its activity is increased by substituting chlorine or alkyl groups on the ring thereby increasing the dissociation of the phenolic OH group and decreasing the solubility of the molecule in water as a result one end of the molecule becomes increasingly hydrophilic and the other increasingly hydrophobic and the molecule as a whole is therefore more surface active (cf next section) The relation between structure and activity of phenols has been reviewed by Suter (1941)

The presence of soap increases the activity of phenol solutions up to a point but if the concentration of soap is too high the phenol is largely taken up in the soap micelles and the disinfectant action drops to that of the soap alone Probably the most widely used disinfectant for discarded bacteriologic material is a mixture of tricresol (mixed ortho meta and para methyl substituted phenol) and soap In a sense the relatively weak action of phenols is of some advantage for this type of disinfection since the requirement for a high concentration reflects a low affinity for organic matter hence the addition of discarded cultures or feces is unlikely to reduce seriously the concentration of free disinfectant

Longer alkyl groups make phenols even more effective than cresol but less soluble Hexylresorcinol phenol with a second OH group and a 6 carbon chain is used as a skin antiseptic and thymol (methyl isopropyl phenol) has such a low solubility that a crystal or two which saturates the solution is a useful preservative of urine enzymatic digests etc

Phenols are capable of inactivating enzymes and in fairly high concentration they denature proteins While the bactericidal action of these compounds has been considered to involve such denaturation of intracellular enzymes they have also been shown like other surface active compounds (cf next section) to cytolyze bacteria

Soaps and Other Surface Active Agents

Soaps (sodium or potassium salts of long chain fatty acids) have long been known to be bacteriostatic and bactericidal especially to gram positive and acid fast organisms They visibly lyse pneumococci Because of the relative resistance of gram negative species oleic acid and similar compounds have been used for the selective cultivation of such organisms as *H influenzae* The action of soap in surgical scrubbing is partly bactericidal as well as detergent but its selectivity prevents it from being a reliable general disinfectant

Certain long chain fatty acids and esters in chaulmoogra oil especially chaulmoogric acid have long been considered to have chemotherapeutic value in the treatment of leprosy but the results have not been striking

Since fatty acids do exist in the animal body the possibility must be considered that they contribute to natural defenses against infection However it seems unlikely that long chain members of the series such as oleic acid can exert an antibacterial action in vivo since they are so tightly bound by serum albumin (Davis and Dubos 1947) Recently Dubos has obtained evidence that short chain acids such as lactic acid may play a significant part in natural resistance

In recent years the investigation of surface active antibacterial compounds has received much impetus from the industrial development of compounds that are generally called synthetic detergents, though other of their surface active properties (wetting emulsifying foaming) are often more prominent than their detergent (cleansing) action

These compounds like soaps are surface active—i e they tend to accumulate in an oriented layer at aqueous interfaces—because they have a hydrophobic portion and a hydrophilic portion The hydrophobic portion of the molecule is predominantly or solely hydrocarbon the hydrophilic portion may be an ionizable group or a nonionic but highly polar structure The nonionic detergents are not particularly antibacterial and in some cases are even good bacterial nutrients they will not be considered here The anionic compounds generally have as their hydrophilic groups a sulfate (RSO_4H) or sulfonate (RSO_3H) group with highly variable R phenols and carboxylates (fatty acids) are also anionic surface active compounds The cationic compounds are usually substituted amines or ammonium or heterocyclic nitrogenous compounds (e g pyridinium) The amines ionize only in acid solution the most valuable compounds are the quaternary ammonium compounds (R_4N^+) which are ionized at all pH s and of these the best have a benzyl and a long chain group as well as two methyl groups (e g Zephiran Phemerol) Cationic germicides are on the market in great variety because of patent rights they all have much the same action some in greater dilution than others

While the anionic detergents are relatively ineffective against gram negative organisms the cationic compounds are practically equally effective against both gram positive and gram negative bacteria and in 10 minutes will completely sterilize large inocula of various species in dilutions of 1 3 000 to 1 30 000 Consequently, they are finding increasing use In concentrations of 0 1 to 1 0 per cent they are especially valuable as skin antiseptics (e g in surgical scrubbing), since they leave a fairly tenacious bactericidal surface film on the skin Considerably higher concentrations of cationic detergents are required to sterilize viruses than bacteria

Detergents, both anionic and cationic combine with proteins and denature them Furthermore anionic and cationic surface active compounds (including the anionic phospholipids) combine with and neutralize each other (Baker et al, 1941a) Hotchkiss (1946) has shown that detergents, in the same high dilutions that sterilize cause cytolysis as evidenced by their extraction of N and P from the cells such extraction does not accompany sterilization by many other agents (e g metal ions formaldehyde) Therefore, it appears that detergents sterilize by disrupting the cell membrane presumably through combining with its proteins and lipids This mechanism may explain why detergents are relatively inactive against viruses which are less highly organized

The antibacterial action of surface active compounds has been reviewed by Baker et al (1941b), Valko (1946), and Glassman (1948)

Alcohol and Other Organic Solvents

The disinfectant action of the aliphatic alcohols increases with chain length up to 8 to 10 carbon atoms above which the water solubility is too low Although ethyl alcohol has long received widest use, isopropyl alcohol which is now available at low cost, has several advantages, being equally miscible with water less volatile more active and not subject to legal restrictions as a potential beverage The disinfectant action of alcohol, like its denaturing effect on soluble proteins, involves the participation of water Ethyl alcohol is most effective in 50 to 70 per cent solution 100 per cent alcohol is a poor disinfectant, in which anthrax spores have been reported to survive for as much as 50 days Solutions of organic disinfectants such as formaldehyde or phenol are less effective in alcohol than in water an indication of a lowered affinity of the disinfectant for the bacteria compared with its affinity for the solvent While the bactericidal effect of ethyl alcohol is negligible at concentrations below 10 to 20 per cent it is bacteriostatic for many organisms at concentrations as low as 1 per cent However the yeasts of alcoholic fermentation can accumulate alcohol in the medium in concentrations up to 12 per cent or more

While bacteria do not flourish in organic solvents such as ether or benzene these compounds are fairly unreliable as rapid disinfectants because of their low solubility in water Protein solutions enzymic digests etc are frequently saturated with toluene or chloro-

form to prevent mold growth Glycerol a polyhydric alcohol is bacteriostatic in concentrations exceeding 50 per cent even though it is an excellent nutrient in lower concentrations for many bacteria it is used as a preservative diluent for vaccines and other biologicals since it is not irritating to tissues

Dyes

A wide variety of dyes have been shown to be bactericidal and bacteriostatic in dilutions extending as high as 1 10^7 (Table 55) There is wide variation in the extent to which staining by various dyes is lethal In general with dyes as with detergents basic (cationic) compounds are more effective than acidic ones Undoubtedly the most valuable result of the systematic study of dyes has been the discovery of sulfanilamide as an antibacterial breakdown product of the dye Prontosil most dyes have proved to be of little value as antibacterial agents except in selective cultivation The main reason for this failure is probably their rapid adsorption onto plasma proteins and tissues Browning reported that the acridine dyes (often called flavines but not closely related to the vitamin riboflavin) are not inactivated by serum and recommended their use in wound antisepsis but they are quite outmoded by modern chemotherapeutics Albert et al (1945) have admirably correlated the antibacterial action of acridine derivatives with their physicochemical properties

Aerosols

The prevention of air borne infection by sterilization of air includes the use of chemical disinfection as well as ultraviolet radiation Robertson (1942 1946) found that a number of glycols such as propylene glycol and diethylene glycol originally used as diluents for other disinfectants to be atomized as aerosols are themselves powerful disinfectants when dispersed in fine droplets even though they are only weak disinfectants in solution They are effective in the air in high dilutions which are nontoxic to man However their practical use is limited by the fact that they are effective in only a narrow range of humidity

Luck (1947) has demonstrated that the action of these chemical aerial disinfectants involves condensation of the glycol from the vapor phase onto the bacteria rather than collision of bacteria with droplets At high humidities the hygroscopic glycols have too low a vapor pressure owing to dilution by water taken up by their droplets at low humidities the bacteria are desiccated and no longer attract glycols It may be noted that the term aerosol denoting a liquid in air emulsion must be distinguished from the same word used as the trade name for certain anionic detergents

Gaseous Disinfectants

Gaseous sulfur dioxide chlorine formaldehyde or ethylene oxide may be used to disinfect articles of clothing etc which cannot

TABLE 55 BACTERICIDAL CONCENTRATIONS (PROLONGED INCUBATION)*

Substance	Bactericidal Concentration			
	S aureus (Gram Pos)		E coli (Gram Neg)	
	Peptone Water	Ox Serum (Heated 56)	Peptone Water	Ox Serum (Heated 56)
Acriflavine (acridine dye)	1 200 000	1 200 000	1 20 000	1 100 000
Anil—48 (quinoline dye)	1 200 000	1 200 000	1 200 000	1 200 000
Brilliant green (basic triphenyl methane dye)	1 10 000 000	1 20 000	1 130 000	1 3 500
Mercurochrome (Hg fluo escein dye)	1 100 000	1 1 000	1 100 000	1 1 000
HgCl	1 1 000 000	1 10 000	1 1 000 000	1 10 000
Phenol	1 250	1 250	1 250	1 250
Chloramine T	1 2,000	1 250	1 2 000	1 250
Iodine	1 10 000	1 700	1 5 000	1 800

1 ml samples of medium containing varying concentration of the disinfectants were given a small inoculum (0 1 ml of a 1 1 000 or 1 20 000 dilution) of a 24 hour peptone water culture The bactericidal concentration is the lowest concentration which after incubation at 37 for 48 hours left no viable cells as shown by subculture

* Modified from Browning C H Medical Research Council System of Bacteriology *1* 20 (1930) and British Medical Journal *1* 73 (1917)

be soaked or subjected to sterilizing temperatures

DYNAMICS OF STERILIZATION

Time Action Curves

When bacteria are sterilized by ionizing radiations the rate of sterilization often follows an exponential (logarithmic) curve $n/n_0 = e^{-kt}$ i e $\log n_0 - \log n = kt$, where n_0 is the initial number of viable bacteria, n is the number after exposure for time t and k is a constant which depends upon the intensity of exposure When the logarithm of the number of surviving bacteria is plotted against time the data fall on a straight line This means that the rate of sterilization is proportional to the number of survivors and hence that the chance that a given cell will be sterilized during a given interval of exposure is independent of the duration of preceding exposure i e the radiation exerts no cumulative effect These results are interpreted according to the target theory as meaning simply that death is caused by the irreversible effect of the absorption of a single ionizing electron in an appropriate location in the cell while absorption elsewhere in the cell is without lethal effect (p 660)

Since the shape of the sterilizing curve fits the target theory it is possible to calculate the target or sensitive volume ' of the viable unit under consideration that volume within which a single ionization is lethal The sensitive volume of one of the smaller viruses so calculated is approximately the same as the known size of the virus particle itself In other words *an ionization anywhere within that* particle is lethal With the larger viruses only a part and with bacteria a very small part of the total volume is sensitive in this sense (Lea 1947) These results lend support to the target theory

The sensitive volume may be considered to represent the total volume of the essential genes i e the group of autoreproductive nucleoprotein *macromolecules of which every* one is essential for cell multiplication Alteration of single ordinary enzyme molecules by radiation is not lethal since each enzyme species is represented by a large number of individual molecules Indeed any lethal action that depended on destruction of enzymes alone probably would have to involve a large proportion of all the enzyme molecules in the cell for as long as the apparatus for making enzymes remains intact even a trickle of building blocks and energy should lead to restoration of the deficient enzymes

While the interpretation presented above *for the lethal action of ionizing radiations* is widely accepted there is no general agreement on the significance of the similar exponential curves obtained with some other agents As early as 1908 Madsen and Nyman observed an exponential curve for the sterilization of bacteria by phenol and a few years later Chick observed a similar curve for killing by heat as also for coagulation of proteins by heat There has been a large controversial literature concerning this "monomolecular killing of bacteria whose kinetics is described by the equation of a monomolecular chemical *reaction This does not mean that only one dis* infectant molecule reacts with the cell but it does mean that the bacterial cell behaves in the presence of a constant concentration of disinfectant as thought the cell were a single unit comparable with a single molecule whose chance of undergoing a lethal reaction is independent of the duration of previous exposure

The simplest physical picture for such a phenomenon would involve the reaction of a disinfectant molecule (or heat) with a single indispensable bacterial molecule This molecule could be pictured as part of the genetic *apparatus of the cell or part of a structural* element such as the cell membrane whose integrity was essential for viability Once a critical reaction had occurred in a membrane there could be a resulting increase of permeability to the disinfectant which would cause a large number of subsequent reactions Or once a change had occurred at a particular point on a long chain of nucleic acid or nucleoprotein one could imagine a *resulting increased re*activity in neighboring regions leading to a spreading disturbance In either event the lethal effect could still be defined as the single chemical hit that resulted in the altered threshold Meanwhile there could be a great number of ' hits with other molecules in the cell whose integrity was not important for viability

This interpretation involves the assumption that the individual cells in a bacterial population are uniformly susceptible to the action of heat or the disinfectant and hence can be treated theoretically in a manner analogous to the statistical treatment of chemical reactions Pharmacologists who are accustomed to study the graded effect of various concentrations of chemicals on cellular functions or on the activ

ity of enzyme solutions are in general loath to accept bacterial death as an all or none effect on a single vital molecule in the cell The alternative interpretation which has been vigorously presented by Clark (1937) assumes that each individual cell has a definite length of survival under a given set of sterilizing conditions and that the gradual effect observed is an expression of the wide variation in susceptibility among the cells of a given population This variation would have a very unusual skewed distribution with a maximal number of cells susceptible to an immediate effect of heat or the appropriate disinfectants and progressively diminishing numbers susceptible to progressively longer exposure This type of distribution though unexpected is not impossible but this hypothesis is further weakened by the observation that the most perfect exponential disinfectant curves have been obtained with spores which might be expected to vary less than vegetative cells

Since the available data are consistent with either theory and no critical experiment has been devised to choose between them it is impossible to state where the truth lies However as our concept of genes acquires more physical and less purely formal character and as the importance of permeability barriers is increasingly recognized the monomolecular theory grows more plausible It has been particularly advocated by Rahn (1945) It must be emphasized that exponential curves are not obtained with all chemical disinfectant chlorine for example gives a sigmoid curve with an initial plateau

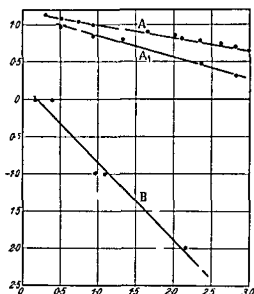


FIG 112 Time concentration curves of disinfection of *S paratyphi* Abscissa log time in minutes Ordinate log concentration in per cent (A) and (A_1) phenol (B) Mercuric chloride From Clark (1937) p 13 after Chick (1908) Note the large shift in disinfection time caused by small shift in concentration of phenol

CONCENTRATION ACTION CURVES

The various concentrations of a disinfectant c required to sterilize a bacterial population in varying time t generally correspond quite closely to a curve which may be fitted by the equation $c\,t = k$ The concentration coefficient (n) of most chemical agents has a value in the neighborhood of 1 in other words the disinfecting time is inversely proportional to the concentration over a wide range of concentration Phenol however has the remarkably high concentration coefficient of 6 (Fig 112) For this reason it is particularly unfortunate that phenol is used as the standard of comparison in practically all the technics devised for the testing of disinfectants including the FDA (Food and Drug Administration) test which is widely used The phenol coefficient furnished by such tests i e the ratio of the concentration of phenol to the concentration of the tested compound necessary to sterilize a given suspension of *Staphylococcus aureus* or *E typhosa* can vary over an extremely wide range up to 50 fold depending upon the time chosen for the end point It also varies widely with the test organism A single value of the phenol coefficient of a compound gives very little information

In practice it is necessary to test disinfectants under conditions (time temperature presence of fecal or other organic matter) which simulate as closely as possible the conditions under which they will have to be used The practical aspects of disinfection and sterilization have been reviewed in detail by McCulloch (1936)

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33

Principles of Chemotherapy Drug-Parasite Interactions

GENERAL ASPECTS AND HISTORY

Chemotherapy is concerned with the use of chemicals (other than those resulting from host defense mechanisms) to inhibit the proliferation of pathogenic organisms within the host Such chemicals must exhibit a selective cytotoxicity i e they must be effective against the parasite at concentrations that can be tolerated by the cells of the host

In the search for methods to control infectious disease the study of chemotherapy began late well after the development of vaccines and antisera To be sure a limited amount of empirical chemotherapy based on primitive folk remedies had been introduced into Europe as early as the 17th century with the importation from South America of cinchona bark (containing quinine) for the treatment of malaria and ipecacuanha root (containing emetine) for the treatment of amebiasis However the development of chemotherapy as a science systematically exploring a variety of compounds and investigating their action depended on the preoccupation—one might say the obsession—of Paul Ehrlich with the notion that chemical specificity could be applied to biologic phenomena

This intense and imaginative investigator received his medical training in Germany at a time when organic chemists were producing a rich variety of synthetic new compounds While still a medical student in 1870 he began his highly productive studies on the use of dyes for the selective staining of tissue components Ten years later he turned his attention to the extraordinarily specific interactions of antigens with antibodies and he formulated the side chain theory which though speculative is quite consistent with modern information on the combining sites on these substances Finally in 1905 he began to pursue the conviction that there must exist synthetic chemicals that like antibodies would have a selective affinity for and hence a selective action on parasitic cells compared with host cells (*nihil agit nisi fixatur*) He developed a number of useful agents against protozoal and spirochetal diseases of which the best known is an organic arsenical arsphenamine (salvarsan) However he did not succeed in developing a similar magic bullet against any bacteria

Pharmacologists of course had long been studying synthetic organic compounds for their possible value as drugs in the treatment of various noninfectious diseases but these studies were naturally concerned with the action of drugs on animal functions Furthermore the rapid advances of immunology also directed attention to the importance of host mechanisms Therefore it is hardly surprising that most students of infectious disease in Ehrlich s time (and long after) believed that in the complex struggle between host and parasite the physician could only hope to help

the host by strengthening his immune mechanisms It was in order to focus attention on *a different approach a direct selective attack* on the parasite that Ehrlich coined the term chemotherapy The meaning of the term is therefore much more restricted than its etymology would suggest

Since the distinction between chemotherapy and pharmacology seems to be worth preserving it is unfortunate that the popular appeal of the modern antibacterial wonder drugs' has caused all sorts of new developments in pharmacology to be labeled as chemotherapy To be sure extending the term to cancer seems to be reasonable since a cancer cell is parasitic even though it arises in the host, but objection must be taken to such expressions as the chemotherapy of arteriosclerosis or that of mental illness

After Ehrlich s death in 1915 his concept of direct chemotherapeutic action was largely replaced by a return to the conviction that drugs could help treat an infectious disease only by stimulating host defenses (cf Dale 1923) This widespread misconception was based on the chemotherapeutic paradox the inability to demonstrate antimicrobial activity in vitro for drugs that were known to be active in vivo The reason is now clear it is the fact that the drugs administered were not the true chemotherapeutic agents but rather were precursors that were converted into these agents by the host For example arsphenamine an arseno compound ($RC_6H_4As = AsC_6H_4R$) was effective only in vivo but many years after its introduction this drug was found to be oxidized in the body into an arsenoxide ($RC_6H_4As = O$) which could then be shown to be active in vitro as well as in vivo

Most of the chemotherapeutic drugs in use today exert their inhibitory action without prior alteration in the body Yet the earlier emphasis on interaction of drug and host however erroneous its conceptual basis was historically important in the development of antibacterial chemotherapy This development was first achieved with Prontosil a drug discovered in 1935 by Domagk at the I G Farben industry in Germany Prontosil proved to be active only in vivo It is indeed fortunate that Domagk in screening a large series of compounds for antibacterial activity chose to test them against streptococcal infection in mice rather than in cultures Otherwise the modern age of antibacterial chemotherapy might have been delayed for years

Prontosil, a red dye is a complex molecule containing a number of groups known to promote fastness to wool, and Domagk had hoped that these groups would promote affinity for bacteria As in so much of the history of chemotherapy, success depended more on good fortune and extensive testing than on a correct preconception For it turned out that the affinity of Prontosil as a dye had nothing to do with its chemotherapeutic action Within a year Trefouel and colleagues at the Pasteur Institute in Paris showed that patients receiving the drug excreted in the urine a simple cleavage product, sulfanilamide which proved to be *the active agent* Therefore prontosil was replaced in therapy by sulfanilamide which in turn served as a model for the synthesis of a number of more active congeners The dramatic effect of these sulfonamides *ushered in what has been called the golden* age of chemotherapy—a 20 year period in which pneumococcus pneumonia and early syphilis have become almost rare diseases in this country the mortality from tuberculosis has been reduced more than 4 fold and effective chemotherapeutic agents have been developed against the large majority of bacterial infections and against rickettsiae Agents against some mycotic diseases are also available but viruses (except for a few of the larger ones) have not yet been reached

It must be emphasized that the central problem of chemotherapy is not the discovery of especially potent antibacterial compounds active at very high dilutions it is rather the discovery of selectively cytotoxic compounds Sulfanilamide, for example, is required in concentrations as high as 10 mg per cent i e 1 10 000 whereas many of the disinfectants described in the preceding chapter are inhibitory in dilutions of 1 1 000 000 Yet these "protoplasmic poisons are chemotherapeutically worthless because they are toxic to the host and because they are inactivated through attachment to various body constituents Hence the mere demonstration that a new product is active in vitro should not lead to premature enthusiasm (For a general discussion of selective toxicity see Albert 1951)

After the discovery of sulfonamides chemotherapeutic research continued to be concerned *primarily with testing synthetic products* of the organic chemist However several years

later attention was largely shifted to anti microbial substances of microbial origin These compounds are called *antibiotics* (Waksman 1947) since the excretion by one organism of an inhibitor of another indicates an antibiotic (i e opposite of symbiotic) ecologic relation ship between the two

Antibiosis among bacteria was recorded as early as 1877 by Pasteur and Joubert who observed sterilization[1] of anthrax bacilli in a contaminated culture There are several dozen later reports of similar observations together with fruitless efforts to utilize the excreted products (cf monograph of Florey et al 1949) One of these reports was that of Flem ing who in 1929 observed that a contami nating colony of the mold Penicillium on an agar plate inhibited the growth of bacteria in its neighborhood Fleming found the active substance too unstable to be useful but 10 years later Chain working in Florey s labo- ratory succeeded in isolating it and he found the purified compound to be reasonably stable and remarkably nontoxic to animals This dis covery of penicillin has led to a search on an enormous scale for other antibiotics

It should be emphasized that the term antibiotic carries no implication of the se lectivity of action that is essential to chemo therapy only a half dozen of the 80 anti biotics described in the Florey volumes can be called chemotherapeutics On the other hand from the standpoint of chemotherapy the dis tinction between antibiotic and synthetic in hibitors is not fundamental as has occurred with other drugs found in nature the recog nition of biologic action is followed by struc tural identification and often eventually by synthesis Thus penicillin and chloramphenı col have already been synthesized

A striking feature of the antibiotics (and especially penicillin) is their effectiveness in remarkably low concentrations (0 01 to 0 1 μg /ml) against many bacteria However this potency becomes less dramatic when con sidered in terms of the number of molecules involved For example a solution of 0 1 μg /ml of penicillin (molecular weight 320) contains 3×10^{-10} mols or $1\ 8 \times 10^{14}$ mole cules per ml the number of molecules per mol being 6×10^{-3} If bacteria are su pended in this solution and if the concentration of drug were the same within the bacteria as in the surrounding medium a coccus of ordinary size (1 cubic micron or 10^{-1} ml) would con tain 180 molecules of penicillin Furthermore the drug actually reaches concentrations in the bacteria many times that in the external medium and Eagle has shown in a compari son of various bacterial strains that the more sensitive ones have greater powers of con centrating the drug

Ehrlich s approach to the search for chemo therapeutics can be described as systematic empiricism the chance discovery of an active compound followed by methodical synthesis of related compounds And despite the ad vance of biochemistry since Ehrlich s time discovery in the field of chemotherapy is still largely empirical Sulfanilamide is a classic ex ample it was discovered as noted above on the basis of an incorrect assumption concern ing the importance of a dyelike affinity and it led to the synthesis of many superior de rivatives years before its mode of action as a competitor of p aminobenzoic acid (PAB) was discovered Similarly after the recognition of the importance of competitive inhibition p aminosalicylic acid (PAS) was introduced as an analogue of salicylic acid which was assumed to be an essential metabolite since it had been shown to stimulate the respiration of mycobacteria Yet this assumption was quite incorrect and PAS turned out to act by competing like the sulfonamides with PAB! Finally when Chain and Florey in 1939 undertook the task of purifying penicillin no one could have predicted that this substance would prove after isolation to be sufficiently stable and nontoxic to become a valuable drug

These successes stand in contrast with the results of more rational biologic and chemical approaches Thus Dubos undertook a search for soil bacteria that utilized other bacteria as nutrients but the inhibitors obtained (gramicidin and tyrocidine) proved to be too toxic for chemotherapeutic use Similarly the synthesis of analogues of essential metabolites has been generally disappointing (p 678) Nevertheless as more is known of the struc ture and the function of the cellular receptors with which drugs can interact chemotherapy should develop an increasingly firm theoreti cal foundation

The search for useful chemotherapeutics has become a big business Students of a pre vious generation were inspired by Ehrlich s

[1] We prefer the term sterilized to killed since bacteria rendered unable to propagate indefinitely have not necessarily lost all other functions associated with life (e g respiration)

persistence in testing 606 compounds before discovering the useful drug arsphenamine But chemotherapeutic tests have now been reported largely by the pharmaceutical industry, *on over 5,000 sulfonamides* or sulfones and 10,000 arsenicals and innumerable microbial *strains have been tested* for antibiotic activity As an index of the impact of chemotherapy on medicine it may be noted that a Nobel *Prize has* been awarded to Domagk for the sulfonamides to Fleming Florey, and Chain for penicillin and to Waksman for *streptomycin*

This chapter will consider the interactions between micro organisms and chemotherapeutic agents with particular emphasis on the *mode of action* of the agent and the mechanism of development of drug resistance These problems can be studied best in vitro Important additional factors must be considered in the animal, including the co operative effect of host defenses, the possibly antagonistic effect of host metabolites to chemotherapeutic action the uneven distribution of a drug in the body the fluctuations of drug concentration with time and the greater variations in the physiologic state of bacteria in different sites in the body But despite these additional complications it should be emphasized that any drug parasite interactions revealed in the relatively homogeneous and well defined environment of a culture tube will be entirely relevant to the problem of chemotherapeutic action in vivo

MODE OF ACTION

Bacteriostatic and Bactericidal Action

An antimicrobial agent is ordinarily recognized through the demonstration that its presence in an adequate concentration prevents an inoculum from yielding visible growth Such tests may involve the outgrowth of colonies on a solid medium or the development of turbidity in a liquid medium The appearance of colonies from single cells requires many generations of growth (of the order of magnitude of 20) the appearance of turbidity may involve a few or many generations depending on the inoculum size

The activity of a drug against a given bacterial strain is determined most precisely by adding varying amounts of the drug in closely graded *steps, to a series of* tubes of liquid medium to which identical inocula are added *the end point is taken* as the lowest concentration that prevents visible growth or the concentration that produces a definite reduction (e g , 50%) in the turbidity reached after a given period of incubation (The turbidity is measured most conveniently with a photoelectric colorimeter) Since borderline concentrations of growth inhibitors usually produce a slowing rather than a complete suppression of growth, the end point observed will depend on the inoculum size and on time Other factors that affect such determinations will be discussed in a later section

The kinetics of inhibition can be studied further by observing the effect of added inhibitor on the growth curve rather than on the ultimate turbidity, of a culture Such tests show that the addition of sufficient chloramphenicol or streptomycin for example, results in an almost immediate cessation of growth the optical density then remaining constant for hours In contrast penicillin causes a marked drop in optical density within an hour indicating lysis (which can be confirmed by microscopic observation) Still another pattern is revealed by sulfonamides which even in high concentrations allow several generations of growth before the optical density becomes fixed

However determinations of inhibitory end points or kinetics fail to reveal (except when lysis is observed) whether the inhibitory effect of the drug is reversible (bacteriostatic) or irreversible (bactericidal) (note spelling) This distinction is important for chemotherapy To make it, two stages of incubation are necessary first a bacterial inoculum is incubated with the drug and then samples taken initially and at varying times during the incubation are diluted and plated for a determination of the viable count

A *bacteriostatic* agent is defined as one whose presence, in a culture that otherwise would be growing reversibly stops growth as a result the number of viable organisms remains constant for hours The reversibility of the inhibition is demonstrated by eliminating the effect of the drug, which is usually accomplished simply by the dilution that occurs in the course of making a plate count In addition, with some drugs reversal can also be achieved by adding an antagonist of the inhibitor (e g

sulfonamides and PAB Hg++ and sulfhydryl compounds)

A *bactericidal* agent in contrast causes a progressive decrease in the number of viable organisms Since the enumeration of viable organisms necessarily involves considerable dilution this means that the agent either is irreversibly attached or has already caused an irreversible damage to the cell It might be noted that bacteriostatic action by definition can be recognized only in a medium adequate for growth whereas bactericidal action can be studied also in simpler menstrua

The distinction between a bactericidal and a bacteriostatic inhibitor is complicated by the fact that some ordinarily bactericidal agents and perhaps all are only bacteriostatic at low concentrations Conversely in the presence of an ordinarily bacteriostatic drug (e g a sulfonamide) the viable population after remaining constant for several hours may decline more rapidly than it does when bacteriostasis is produced with the same organisms by exhaustion or deficiency of the medium

In addition the line between a reversibly and an irreversibly inhibited cell is not altogether sharp whether a damaged cell will be counted as viable or nonviable depends partly on the culture medium Thus at a given moment the number of observed survivors of bactericidal action is generally larger if the enumeration is made in a rich rather than a simple medium The damage to the surviving cells can also be shown by their unusually long lag period before growth in fresh medium is resumed For chemotherapy it is even more important that in resurrecting these damaged bacteria the animal host is sometimes less effective than the usual culture media Thus a streptococcal culture exposed to penicillin has been found (Eagle) when still containing cultivable organisms to have lost all virulence for mice even though ordinarily a single viable organism initiates infection

Despite these qualifications it is useful to distinguish between a bacteriostatic drug which produces an essentially constant viable count for at least several hours and a bactericidal one which causes a rapid decline in the viable count (i e by logarithmic units per hour) From the point of view of chemotherapy it makes a great difference whether after a limited period of exposure to a drug growth is resumed by most of the bacteria initially present or by only a tiny fraction of them This difference is probably responsible for the fact that optimal therapy with the bacteriostatic sulfonamides requires the continuous maintenance of adequate concentrations in the body fluids whereas the same requirement does not extend to such bactericidal drugs as penicillin and streptomycin

The greater efficacy of penicillin and streptomycin compared with sulfonamides suggested at first that bactericidal chemotherapeutics might be inherently superior to bacteriostatic ones which leave the sterilization of an invader to the host defenses However this generalization now seems to be questionable since some very effective antibiotics (chloramphenicol tetracyclines) are bacteriostatic It therefore seems likely that the value of sulfonamide chemotherapy is limited by special features of the action of these drugs (p 677) rather than by the fact that this action is only bacteriostatic

Various bactericidal agents differ in the conditions required for their action Penicillin has the unique property of being bactericidal only under conditions that allow growth (Hobby) Streptomycin in contrast requires active metabolism but not necessarily growth (Davis) and disinfectants described in the preceding chapter are active even in distilled water their irreversible reactions with cell constituents apparently not requiring the co-operation of metabolic processes

The metabolic activity required for the action of penicillin and streptomycin helps explain why these compounds though actively bactericidal rarely eradicate an infection in a single dose Even in the test tube where most of the population is sterilized within an hour or two by either of these drugs a few cells remain viable for long periods These persisters are presumably in a dormant physiologic state in which the metabolic processes required for sterilization are suppressed These cells are quite different from drug resistant mutants which are not dormant but continue to multiply in the presence of the drug One might anticipate that in the body the numbers of persisters would be increased in regions where extensive microbial growth had suppressed metabolic activity by exhausting the supply of nutrients or otherwise modifying the environment

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fied by enzymatic studies on the conversion of PAB to an essential coenzyme

PAB was found to be extremely potent it completely reversed the action of 200 times its concentration of sulfanilamide However the subsequent development of more powerful PAB competitors showed that such a high ratio is not a necessary characteristic of competitive inhibition thus the competitive ratio of sulfathiazole to PAB is about 10

PAB was soon proved to be an essential metabolite In the nutrition of certain bacteria it is required as a vitamin In most others it is not a required nutrient but is synthesized by the cell from constituents of the medium A third group are unable either to synthesize or to utilize PAB they require instead a preformed PAB derivative (as do mammals) The first two groups of organisms which possess a PAB-conjugating enzyme are sensitive to sulfonamide inhibition though the concentration required varies widely the third group which lack this enzyme are insensitive to sulfonamides No organisms are known that normally function without PAB in any form

Several PAB derivatives (the folic acid group) have now been found in nature and more may well exist This group includes folic acid (pteroyl glutamic acid) and the closely related citrovorum factor (folinic acid leucovorin formyl tetrahydrofolic acid) These two vitamins are interchangeable in the nutrition of certain animals and *micro-organisms* other organisms have a specific requirement for one or the other These *vitamins in turn* function as coenzymes in the formation or the transfer of a one carbon fragment essential for the synthesis of a group of metabolites *includ*ing methionine serine purines and thymine

METABOLIC FUNCTION OF PAB

Sulfonamide
PAB ——→ Folic acid (pteroylglutamic acid)
Citrovorum factor etc
– – – | – – Aminopterin
Coenzyme F — Methionine, Serine, Purines, Thymine, Pantothenate
(Tranformylation reactions)

It should be possible to restore growth not only by supplying a substrate which reverses the inhibition of a reaction but also by supplying a product which eliminates the need for the reaction Under the latter circumstances reversal of growth inhibition should be noncompetitive i e the amount of the product required to restore growth should not increase with increasing concentrations of the inhibitor With sulfonamides it would be expected that inhibition should be reversed noncompetitively both by the direct products of PAB metabolism the folic acid group and also by a complete set of its indirect products the metabolic building blocks whose synthesis depends on folic acid Indeed such an effect has been observed

It should be noted that the available members of the folic acid group reverse sulfonamide inhibition in only a few bacterial species It is not known whether the remaining species convert PAB into a derivative different from the available ones or whether the bacteria are impermeable to these compounds

This analysis of PAB function has clarified certain aspects of sulfonamide action For one thing it is now clear that the chemotherapeutic value of sulfonamides or other PAB competitors rests on two special circumstances (1) The cells of the animal host do not utilize PAB as such and hence are not susceptible to inhibition by its analogues This fact accounts for the ability of sulfonamides to exhibit the desired selectivity (2) Most bacterial species cannot use the PAB derivatives *that are available* in the animal hence sulfonamides can be effective in vivo as well as *in vitro* Those species (e g enterococci) that can utilize folic acids are quite unresponsive in vivo to sulfonamides

The above analysis also clarifies certain limitations of the chemotherapeutic value of sulfonamides (1) Certain of the noncompetitive antagonists of sulfonamides especially the amino acids are available in body fluids Because of this circumstance the level of drug required for inhibition in vivo is somewhat higher than that observed in vitro in a simple medium (2) The complete set of these noncompetitive antagonists can be released by autolysis this process can account for the unpleasant fact that sulfonamides are completely

The fact that penicillin requires growth for its sterilizing action also explains the observation that under some circumstances penicillin plus a bacteriostatic agent is less effective in vitro and in vivo, than penicillin alone (Hobby, Jawetz) This fact also explains why the rate of bactericidal action of penicillin on some bacteria (e g enterococci) is paradoxically decreased at excessive concentrations (Eagle, 1948) presumably the drug at these concentrations reversibly inhibits some reaction which in turn is required for irreversible action by the drug In the ordinary range of dosage however this effect is unlikely to be clinically important *Streptomycin shows no* such paradox the bactericidal rate increasing with concentration over a wide range

Valuable cross fertilization between chemotherapy and microbial genetics has been provided by the use of penicillin for the isolation of nutritionally exacting (= auxotrophic) mutants of bacteria i e strains that require a given metabolite because they are genetically blocked in a reaction in its biosynthetic pathway Such mutants can be screened readily by exposure to penicillin in a minimal medium adequate for the wild type parent organisms the latter proliferate and are consequently sterilized while the mutants which cannot grow in this medium survive (Davis Lederberg and Zinder) These mutants in turn are valuable tools in the study of biosynthetic paths whose analysis is important for an understanding of chemotherapy as well as of cell physiology

A Bacteriostatic Mechanism Competitive Inhibition (Sulfonamides)

Bacteriostatic action is brought about by the reversible inhibition of one or more reactions that are essential for the growth of the cell

Reversible inhibition of an enzyme can be caused by a compound that resembles the substrate sufficiently to compete with it for attachment to the active site of the enzyme But while this mechanism has received most attention in recent years, it is not the only one that should be considered Thus reagents that combine reversibly with sulfhydryl groups can inactivate many enzymes and such inhibitions are not competitively reversed by the substrate Such noncompetitive mechanisms of inhibition might be responsible for the action of some bacteriostatic agents, such as chloramphenicol for which no reversing agents are known However since competitive mechanisms are much better understood and since they involve a fruitful principle, they will be considered here in some detail

Though sulfonamides are responsible for bringing the concept of competitive inhibition into prominence in chemotherapy and pharmacology the concept arose much earlier in biochemistry Originally, it was developed through a study of carbon monoxide poisoning which was found to be due to competition between this compound and oxygen for the same sites on hemoglobin and related molecules the affinity of each combination is expressed by a characteristic dissociation constant In mixtures of these two compounds the relative combination of hemoglobin with each depends on both the ratio of their concentrations and the ratio of their affinities for hemoglobin increasing concentrations of either competitor will increasingly displace the other

Similar phenomena have been observed with enzymes A clear example is the demonstration by Quastel and Wooldridge in 1927 that the action of succinic dehydrogenase on succinic acid is inhibited by malonic acid and the inhibition can be reversed by increasing the succinic/malonic ratio The two compounds differ by only one carbon atom and apparently both compete for the same site on the enzyme though only one undergoes dehydrogenation as a result of this combination It is characteristic of such competitive inhibition that as the concentrations of the inhibitor and its antagonist are varied the threshold of inhibition continues to be observed at a fixed ratio of the two compounds

In 1940 D D Woods extended the demonstration of competitive inhibition from extracted enzymes to growing cells Analyzing the antagonism of yeast extract to the inhibition of bacterial growth by sulfanilamide he identified the antagonist as *p* aminobenzoic acid (PAB) and showed that the relation between this compound and sulfanilamide was competitive As in other cases of competition the inhibitor and its antagonist had a close structural resemblance (see structural formulae p 687) It was suggested that PAB is a substrate of some unknown *essential metabolic* reaction and that sulfanilamide blocks this reaction by interfering competitively with the temporary attachment of PAB to the enzyme involved This conclusion has since been veri

citing chapter in the study of drug action (Matthews 1958)

A Bactericidal Mechanism Unbalanced Growth (Penicillin)

It was noted in the preceding chapter that chemical disinfectants are in general either substances that dissolve membranes or chemically reactive substances that denature macromolecules neither class requires cellular activity The bactericidal chemotherapeutic agents (e g penicillin streptomycin) do not fall into these categories but require metabolic activity Only recently was a firm basis established for understanding why certain ways of interfering with the metabolism of a cell should have an irreversible lethal effect

This understanding arose from observations on the effect of depriving bacteria of various required building blocks the deprival could be achieved either by mutation or by addition of an antimetabolite When the cells are deprived of an amino acid a ribonucleic acid component or a vitamin growth ceases sooner or late (as shown by measurements of optical density) but the cells remain viable for many hours However S S Cohen (1954) found that when bacteria are deprived of thymine a component unique to DNA incubation in an otherwise adequate medium is rapidly lethal What happens is that the cells continue for a time to make protein and ribonucleic acid but can make no DNA (or at least no normal DNA) and after a sufficient amount of this unbalanced growth the damage to the cell is irreversible

Another kind of unbalanced growth has turned out to be the explanation for the action of penicillin an interference specifically with the formation of cell wall This conclusion is supported by two sets of observations First it is known that lysozyme dissolves the wall of certain bacterial species and as a result in ordinary media it leads to lysis However in a hypertonic medium such as 20 per cent sucrose the action of lysozyme leads to the formation of a bacterial protoplast a spherical osmotically fragile cell consisting of the cell membrane and its entire contents but lacking a wall (Weibull) Building on these observations together with the knowledge that growth of susceptible bacteria in the presence of penicillin leads to lysis Lederberg (1957) showed that growth in the presence of penicillin plus 20 per cent sucrose results in the formation of protoplasts It can be concluded that penicillin interferes with cell wall formation as a result the growing cell bursts out of the wall as a protoplast It is to be noted that this action is quite different from that of lysozyme which attacks already existing cell wall

The other evidence is biochemical in nature In 1949 Park and Johnson showed that under the influence of penicillin certain bacteria accumulate in the medium a compound containing a uridine nucleotide an acetyl amino sugar and a peptide of 3 amino acids (DL-alanine D glutamate and lysine) It was discovered subsequently that this sugar and peptide are found in quantity in separated bacterial cell wall These findings led to the conclusion (Park and Strominger 1957) that the compound is an intermediate in cell wall formation the nucleotide acting as an activator for the portion to be incorporated into the wall Penicillin interferes with this incorporation It is not yet known at just what biochemical site penicillin acts it might interfere with an enzyme that transfers the building block from the nucleotide to a postulated receptor in the wall but the accumulation could equally be explained if penicillin interfered with the formation of the postulated receptor

These findings suggest that bactericidal action may result from the action of any inhibitor that allows most parts of the cell to grow while preventing normal growth of a vital unit such as a chromosome a membrane or the wall

ANTIBACTERIAL SPECTRUM

Sensitivity and resistance are quantitative rather than qualitative attributes each chemotherapeutic tested at a high enough concentration can inhibit practically all strains of bacteria However inhibitory effects observed only at excessive concentrations are not chemotherapeutically valuable and they may not involve the usual mode of action of the drug It is therefore at a chemotherapeutically practicable concentration that one determines each drug's antibacterial spectrum the species and even strains within a species that are susceptible to it

ineffective in purulent exudates and other sites of extensive tissue destruction (3) At least some species (e g streptococci) can store large amounts of a PAB derivative and hence continue to proliferate for as many as 6 generations (e g 64 fold multiplication!) after the adddition of even a large excess of sulfonamide The onset of sulfonamide bacteriostasis is thus a delayed one

Woods' discovery of the PAB sulfonamide relationship suggested a rational approach to research in chemotherapy the synthesis of structural analogues of essential metabolites The next few years saw the synthesis of hundreds of analogues of other vitamins and also of amino acids purines and pyrimidines (Woolley 1952) Unfortunately though many of these antimetabolites (= metabolite analogues) inhibit the growth of micro organisms in vitro no practical antibacterial chemotherapeutic has resulted from this work In the treatment of leukemias however some analogues of folic acid purines and pyrimidines have shown striking effects

The main limitation of this approach probably lies in the fact that the compounds used as models for these syntheses have been metabolites common to micro and macro organisms in consequence the host possesses the same susceptible reactions as the parasite and also supplies the reversing metabolite in the fluids in which the parasite grows This consideration suggests that it might be particularly useful to model the analogues after metabolites that have like PAB the property of being restricted to microbes Unfortunately few such metabolites are known One *p* hydroxybenzoic acid (POB) was revealed with the aid of bacterial mutants This development led to an explanation of the already known and surprising fact that PAB not only is a metabolite but in large doses is an effective chemotherapeutic against rickettsiae This effect was found to be due to competition with the structurally similar metabolite POB However analogues of POB have not proved to be useful against bacteria

Despite the limitations noted it would be premature to abandon the search for chemotherapeutics among synthetic analogues Encouragement is offered by the species specificity observed with some competitive inhibitors Thus competitive analogues of PAB can be constructed not only by substituting a sulfonamide group for the carboxyl radical but also by substitutions for the amino group or a ring hydrogen Among such nonsulfonamide analogues only one *p* aminosalicylic acid (PAS), is clinically valuable This inhibitor shows the surprising property of being much more effective than sulfonamides against tubercle bacilli, and yet less than 1/100 as active against colon bacilli Similar differences in sensitivity might well exist between host and parasite and hence might furnish a basis for selective inhibition

Nevertheless it is significant that the antibiotics that have largely replaced sulfonamides are not reversed in their action, competitively or noncompetitively, by known compounds or even by complex biologic extracts Therefore eventually competitive inhibition may cease to be of major chemotherapeutic importance It has seemed to be desirable however to discuss this chemotherapeutic mechanism in detail, since it is the best understood Furthermore this development has contributed to biochemistry as well as to chemotherapy, for the quantitative study of competitive and noncompetitive reversal of inhibition by metabolite analogues has provided a valuable tool for studying paths of biosynthesis However the interpretation of such inhibitory relationships is not always unequivocal since it has been shown recently that structural analogues can compete with each other not only at enzymes within a cell but also at specific permeation units in the cell membrane Hence a reversing agent is not necessarily a substrate it could also act by interfering with penetration of the inhibitor Conversely in some cases an antimetabolite has been shown to act by interfering competitively with the penetration of a required nutrient

A new aspect of competitive inhibition that has come to light recently is the biosynthetic incorporation of antimetabolites For many years it was assumed that analogues could only jam the lock ' More recently the use of isotopically labeled compounds has revealed that many antimetabolites are also extensively incorporated by bacteria Included are analogues of amino acids of nucleic acid components and of vitamins In some cases considerable incorporation up to 20 per cent or more of the content of the normal metabolite is consistent with growth and viability in other cases growth proceeds for a generation or so but then grinds to a halt In such a case it appears that the analogue is not directly blocking a reaction sequence but is being incorporated into a more complex derivative such as protein or nucleic acid which then fails to function This promises to be an ex

Only within the past decade however has it become generally recognized that the two processes have many features in common not only are their effects inheritable but both changes occur spontaneously with a low frequency (of the order of magnitude of once in a million to a billion cells) and are increased in frequency by certain physical agents (ultraviolet or ionizing irradiation) or certain chemicals (e g nitrogen mustards) The resemblance is further emphasized by recent evidence that bacteria have much the same genetic apparatus (including chromosomes) as do the cells of higher forms and exhibit in some cases the ability to undergo a recombination of hereditary properties (including acquired drug resistance) suggestive of sexual fusion It therefore appears justifiable to refer to undirected inheritable bacterial changes as mutations

Spontaneous mutation followed by natural selection is considered to be the mechanism of evolution in the biologic kingdom Even before the discovery of mutations biologists had largely abandoned the alternative Lamarckian view that organisms can inherit characteristics acquired by a specific adaptation to the environment Viewing the development of an inheritable change in a bacterial culture as an example of evolution one would expect the role of the drug in producing a resistant culture to be simply that of providing a selective environment in which no organisms could proliferate except drug resistant mutants that might arise

But the 19th-century experiments that defeated Lamarckism involved only characteristics (e g mutilations) acquired by somatic cells and hence lacking evident means of influencing the germ cells which govern inheritance In bacteria however there is no distinction between germ cell and somatic cell It is therefore conceivable that a drug having penetrated into a bacterial cell might somehow bring about a mutation to drug resistance in a directed manner The fact that resistance arises only in a tiny fraction of the population might seem to suggest a spontaneous origin but the nature of the experiment does not exclude a directive role of the drug since recognition of the resistant cells requires exposure to the drug—and it might be during this exposure that the mutation first occurs

Definitive evidence on the origin of drug resistance was furnished by a statistical approach (fluctuation analysis) designed by Luria and Delbruck (1943) to study bacteriophage resistance and subsequently applied by Demerec to drug resistance In this type of experiment a number of small inocula containing no resistant mutants are seeded in identical tubes of medium and incubated when growth is complete the number of resistant cells in each tube is determined by plating in the presence of the drug If the resistant mutants arise only during exposure to the drug the different tubes should give identical results except for a small predictable statistical variation in sampling If on the other hand the mutations have arisen during growth of the culture before exposure to the drug the tubes will differ from each other in the time of occurrence of the first chance mutation to resistance and hence in the number of generations developing subsequent to this mutation in consequence the size of the family of resistant progeny descended from this resistant cell will vary and hence the number of such cells finally present will fluctuate more widely In actual experiment wide fluctuation was observed a few jackpot tubes (those with a very early mutation) having a very large number of mutants These results showed conclusively that resistant mutants had arisen before exposure to the drug

Fluctuation analysis shows how an ingeniously designed experiment can furnish a decisive conclusion that replaces earlier opinions formed on the basis of intuitions and analogies —often the only available basis for *answering* complex medical and biologic questions but hardly a scientific one Even more direct evidence for spontaneous origin of drug resistant mutants has since been obtained by other workers (Newcombe Lederberg) who showed that after growth of bacteria densely spread on a plate of medium *without drug* resistant cells detected by subsequent transfer were present in clusters Some investigators (e g Hinshelwood) still believe that drugs play a directive role but their evidence does not include the critical test of fluctuation analysis

The present state of the problem can be summed up by saying that spontaneous mutation plus selection has been demonstrated in some cases of drug resistance and drug directed mutation in no case but specifically directed mutations are still theoretically pos

With the presently increasing variety of available chemotherapeutics it has become important in many cases of infectious disease, to determine the sensitivity of the organism to various agents This is conveniently accomplished by testing a plate, inoculated with the isolated organism, for inhibition by paper disks impregnated with various drugs Such disks are commercially available and this crude test is often adequate (especially for staphylococci which vary so widely in their pattern of sensitivity) However, more precise determinations based on testing against serial dilutions of the drugs are sometimes desirable in handling infections which are difficult to treat (e g subacute bacterial endocarditis or chronic urinary tract infections)

Susceptibility to a drug has not been successfully related to other known properties of various organisms Thus while the correlation of penicillin sensitivity with a positive Gram stain is often stressed exceptions such as the highly sensitive gram negative *Neisseriae* disprove this rule

The degree of sensitivity to a given chemotherapeutic clearly involves factors other than the qualitative presence or absence of the reaction or structure affected by it There is evidence that in some cases resistant species are less permeable to the drug Another possibility is that differences in resistance between species as between parental strains and mutants with acquired drug resistance may arise from individual genetic fingerprinting of enzymes i e production of enzymes that differ in their affinity for an inhibitor, though they carry out the same reaction

DRUG RESISTANCE

Occurrence

Ehrlich and his collaborators soon after initiating the development of modern chemotherapy discovered the unexpected phenomenon of drug fastness and made a number of fundamental observations on it (1) the degree of resistance varied from one strain to another (2) there was no cross resistance between members of the different chemical series studied (fuchsin dyes azo dyes arsenicals) (3) the members of a given series in contrast showed cross resistance presumably due to affinity for the same receptors

These discoveries made with trypanosomes in mice were largely neglected until the same phenomena were encountered again with antibacterial chemotherapeutics When bacteria are grown in the presence of a moderate concentration of any chemotherapeutic strains with at least some degree of resistance quite regularly emerge Resistant bacteria similarly tend to appear in treated patients or animals whose infection is not rapidly eradicated Furthermore resistance of the bacteria continues to be inherited during subsequent cultivation, even in the absence of the drug

With most drugs the degree of resistance that can be developed in vitro in a single exposure is quite small the progeny being at most from 2 to 4 times as resistant as the inoculated cells By successive transfers with increasing drug concentrations high levels of resistance can often be built up However with streptomycin high levels of resistance appear not only in this gradual manner but also occasionally much more dramatically some species producing in a single step colonies with a thousand fold increase in resistance

Drug resistance presents two major theoretical problems a genetic and a physiologic one (1) How does the inheritable difference between sensitive parent and resistant progeny arise? (2) What is the biochemical nature of this difference?

Evidence for Mutational Origin of Resistance

Bacterial variations with few possible exceptions fall into two classes—physiologic and genetic Physiologic adaptations to a changed environment involve all the cells in a culture and are noninheritable being reversed during subsequent growth in the original environment genetic changes involve only a tiny fraction of the cells in the original population and are inheritable being transmitted from generation to generation of the offspring of the changed cells even during growth in the original environment Drug resistance belongs to the latter class which also includes many inheritable changes in a variety of other characteristics such as morphology nutritional requirements virulence etc

Inheritable bacterial variations resemble the mutations of higher organisms as Beijerinck pointed out within a few months after the discovery of mutations by DeVries in 1900

5 Increased destruction of the inhibitor (or decreased conversion of an administered compound into a more active inhibitor)

6 Formation of an altered enzyme with de creased affinity for the inhibitor or with in creased relative affinity for the substrate com pared with a competitive inhibitor

7 *Decreased permeability of the cell (or of* subcellular units) to the inhibitor

The first mechanism involving an alterna tive metabolic path has long been a specula tive favorite, especially since drug resistance arises by mutation and since certain other mutations have an all or none effect on the formation of a given enzyme However it has become abundantly clear that the biochemical consequence of mutations is by no means re stricted to such an all-or none effect and so this mechanism has lost much of the basis for its appeal Furthermore there are reasons to *doubt whether this mechanism occurs at all* For in a biosynthetic sequence proceeding from compound A to C via B the appearance of a new route bypassing B surely would have to involve the insertion of more than one new enzyme in the sequence Of course it is pos sible that one or more of these enzymes though new in the sequence might already be pre ent in the cell for other purposes The theoretical objection to this mechanism is therefore not absolute but it should be added that the mechanism has not been clearly dem onstrated in any case Furthermore this mech anism could not readily account for the graded degrees of resistance that are generally ob served

Mechanism 2 increased concentration of a competitive metabolite has also had wide spread appeal ever since sulfonamides were shown to act by competing with *p* aminoben zoate (PAB) However in the few cases where increased formation of PAB has been demon strated the effect has been too slight to explain more than a trivial increase in resistance Furthermore despite much effort none of the antibiotics has been shown to act by com peting with a metabolic intermediate

The next two mechanisms increased con centration of the enzyme or decreased require ment for its product could also hardly be expected to produce more than a modest increase in resistance

Mechanism 5 destruction of the inhibitor has been demonstrated in many (though not all) penicillin resistant strains and it is the mechanism found in the penicillin resistant staphylococci that are encountered so fre quently in hospitals today It should be noted that resistance resulting from such penicilli nase production is unusually dependent on population density With most mechanisms the resistance is inherent in the individual cell and the level observed is much the same for *single cells on an agar plate or for relatively* large inocula In the case of a penicillinase producer however whether or not the culture survives will depend on competition between the rate at which the drug sterilizes the bac teria and the rate at which the bacterial en zyme destroys the drug Hence an isolated penicillin producing cell may show little or no resistance while a large inoculum which pro vides a large amount of enzyme to the culture will show much resistance Clinically this form of resistance is effective because of the nature of staphylococcal infections in which high bacterial population densities are attained in localized areas

Penicillinase producing mutants have proved to be practically impossible to isolate in vitro probably because the original mutant cell ap pearing in a population is not resistant as a single cell This finding as well as epidemio logical evidence supports the view that the penicillinase producing staphylococci seen in patients generally represent cross infections rather than the emergence of a resistant mutant from a sensitive strain

Mechanism 6 the formation of an enzyme with altered affinity has not been directly proved in any case but there is substantial indirect evidence that this is the mechanism in some sulfonamide resistant strains (Davis and Maas 1952) Furthermore it has been shown that mutations can lead to changes in other properties of proteins such as sensitivity to thermal denaturation or solubility and electrophoretic mobility (sickle cell hemo globin) It would be most surprising if the affinity of an enzyme for a drug should not also be subject to alteration by mutation A close parallel with this postulated variation is provided by the hemoglobins found in the ani mal kingdom which vary over a 200 fold range in their relative affinity for their normal substrate (oxygen) and for a competitor (carbon monoxide)

The last mechanism decreased permeabil ity was suggested early by Ehrlich but it

sible Indeed though the principles of classic genetics have been applied recently with striking success to the phenomena of bacterial variation the possibility of other mechanisms in bacteria should not be rigidly excluded Thus in morphogenesis (embryologic differentiation) the environments of the various parts of the unfolding organism somehow guide the differentiation yet these "adaptive" changes are stably inherited and are essentially irreversible on change of environment (e g tissue culture) Similar phenomena may yet be found in micro organisms

There is little doubt that spontaneous mutation to drug resistance occurs at much the same rate in the patient as in the test tube In either circumstance however the speed of emergence of a predominantly drug resistant population depends not only on the rate of mutation but also on the efficiency of selection In the homogeneous environment of the test tube the possibility of sharp selection, with survival or proliferation only of mutants resistant to a given concentration of drug allows precise quantitative experiments In the experimental animal or patient however selection will be affected by a variety of other factors These include variations with respect to time and to region of the body in drug concentration bacterial population density rate of multiplication rate of bactericidal action and elimination of mutants and non mutants by host defenses It is easy to see how these complicated interactions of drug parasite and host can reduce the predictability of the results

One argument against the mutational theory of drug resistance has been based on the observation that resistance is not always maintained indefinitely on cultivation in the absence of the drug However this observation can be explained by two facts that many resistant strains of bacteria grow more slowly than the parent strain and that most mutants revert at an appreciable rate by a second mutation to strains that behave like the original parent (wild type) Therefore slow resistant mutants can be expected to give rise to occasional faster, more sensitive reversions Since even a tiny differential growth rate will lead during prolonged cultivation to a practically complete shift in the composition of a population one would expect these resistant cells to be replaced eventually in the absence of the drug by sensitive ones Similarly some resistant strains show decreased virulence and are replaced, on animal passage by more virulent reversions

[2] Acriflavine has been shown in yeasts (Ephrussi) and treptomycin in algae (Provasoli and Hutner) to induce the loss of self reproducing cytoplasmic units essential for certain important but not indispensable *metabolic functions* (oxidation and photosynthesis respectively) However these specific drug induced inheritable changes are quite different from drug resistance since (1) they are not accompanied by an increase in resistance and (2) they occur in most or all of the cells in the population rather than in very few

Biochemical Mechanisms of Resistance

The origin of inheritable drug resistance having been traced to mutation plus selection it remains to determine what is the phenotypic (physiologic biochemical) change in the cell that results from the mutation and is recognized as drug resistance

Ehrlich and subsequent workers showed that drug resistant trypanosomes had a lower arsenic content after exposure to an arsenical than did the more sensitive parent strain Similar differences have been demonstrated using isotopically labeled penicillin with a strain resistant to this drug (Rowley) Ehrlich interpreted his results as showing a decrease in the affinity of the cellular receptors for the drug However, the findings could just as well be due to decreased permeability to the drug or to a lowering in the number of receptors In evaluating such results it must be remembered that not all of the drug present in a cell is necessarily significant it is possible that a large fraction is bound at sites that have no relation to the chemotherapeutic action

In approaching this problem it must be emphasized that at a biochemical *level there is* no single mechanism of drug resistance If various mutations can give rise to an increment of resistance through various mechanisms it would be expected that the inhibitor would select them all A list of theoretically possible mechanisms follows and it is not surprising that examples of a number (though not all) of them have been demonstrated

1 Alternative metabolic pathway bypassing the inhibited reaction

2 *Increased concentration* of a metabolite that antagonizes the inhibitor

3 Increased concentration of the enzyme that the drug inhibits

4 *Decreased requirement for* a product of the inhibited metabolic system

the hosts may lead by a selective process to widespread distribution of strains resistant to that drug And since many (though not all) resistant strains of bacteria appear to be as virulent as their sensitive parents they persist in the population

A striking example of response to such an ecologic influence is presented by the gonococcus an organism that is particularly susceptible to this type of selection because its transmission is restricted to human beings In New York City for example the proportion of cures of gonorrhea achieved with sulfonamides dropped from about 90 per cent in 1936 to about 30 per cent in 1942 Had other drugs (e g penicillin) not become available the chemotherapy of gonorrhea would have faded away At present a similar spread of streptomycin resistant tubercle bacilli appears to be taking place to a much smaller extent

One of the major problems in infectious disease today is the increasing prevalence of staphylococci resistant to penicillin and also to other agents such as erythromycin and novobiocin Furthermore urinary tract and other infections are frequently found to be caused by gram negative rods that are resistant to a variety of chemotherapeutics In these cases in contrast with the tubercle bacillus and perhaps the gonococcus it is not at all clear that the resistant organism arose as a mutant in a patient under treatment Indeed the available evidence favors the view that these are naturally resistant wild type organisms already present in the pre antibiotic era but now becoming more prevalent

One obvious reason for this prevalence is a tendency in hospitals to let chemotherapeutics serve as a substitute for good aseptic and hygienic technics A second reason possibly very important but difficult to prove is the creation of an empty ecologic niche (e g in carriers) into which resistant strains move after sensitive ones have been eliminated by chemotherapy

It should be emphasized that such natural resistance is not really a problem of drug resistance in the sense of being a genetic problem it is simply an expression of the fact that each chemotherapeutic has limits to its useful antibacterial spectrum and in some cases the limit falls within a species When we find that streptomycin is effective against *E coli* but not against Pseudomonas we dismiss this as an inevitable limitation of spectrum but when we find that penicillin is effective against some strains of Staphylococci but not against others we worry about drug resistance!

The fact that a given species is drug sensitive as encountered in the wild state in nature even though it has continually given rise to resistant mutants in the past implies that the sensitive strain has greater evolutionary survival value in the absence of the drug than its resistant derivatives Otherwise the species would have become drug resistant as many other species have done Hence one could reasonably predict that if streptomycin were no longer used in the world streptomycin resistant tubercle bacilli would eventually disappear However other species such as staphylococci appear able to survive well in either a drug resistant or a drug sensitive form for penicillin resistant strains were encountered (though less frequently) even in the early tests of the drug

It is of interest to note that the emergence of drug resistant cells has also been observed to limit the chemotherapy of a neoplasm (leukemia) with folic acid analogues The mechanism of this emergence appears to be the same as that occurring in bacteria i e mutation plus selection

While this section has emphasized drug resistance as a limitation on the value of chemotherapy it should be noted that in most acute bacterial infections this is not a serious problem

Methods of Preventing the Emergence of Drug Resistance Combined Therapy

One path to therapeutic escape is through the development of successive small increments of resistance and theoretically this process can be blocked by maintaining drug concentrations in the body high enough to provide a margin of safety i e to continue to inhibit the first stage resistant mutants However this procedure offers no protection against one step mutants to a high level of resistance For example in *H influenzae* meningitis treated with streptomycin (Alexander and Leidy) it is a matter of chance whether or not resistant cells are already present at the start of therapy In either case the initial effect of the drug will be dramatic but if a resistant cell is present a relapse will occur regardless of dosage

was difficult to accept as a plausible mecha nism so long as our thinking about cell per meability was dominated by a physicochemical approach to the kinetics and the thermo dynamics of a passive membrane, with the cell viewed as a sort of cellophane bag filled with enzymes With such a naive model it would be difficult to explain how quantitative de creases in permeability could lead to corre sponding decreases in uptake for with a non metabolizable drug decreased permeability should lead to a decreased *rate* of approach to equilibrium but not to a change in the dis tribution at equilibrium In the past few years however our picture of the permeability prop erties of bacteria has altered drastically The cell membrane now possesses a variety of per meation systems each stereospecific for a structurally related group of substrates and the number of units of each kind per cell affects not only the rate of equilibration be tween intracellular and extracellular substrate but also the value of the ratio reached at equilibrium (This development has arisen not only from direct studies of the intracellular concentration of various substances but also from studies of the phenomenon of cryp ticity —i e *the fact that certain enzymic* activities can be demonstrated only after dis ruption of the cells) It therefore becomes easy to see how different degrees of perme ability to a drug could yield different ratios between the intracellular and the extracellular concentration

Evidence for such a mechanism has been obtained (Kushner) in the case of chloram phenicol resistance in Pseudomonas since in tact resistant cells were found to be less sus ceptible than the sensitive parental cells to inhibition of oxidation of a variety of sub strates whereas disrupted cells of the two strains failed to show this difference

Drug Dependence

Miller and Bohnhoff (1947) discovered the development of mutant strains of meningo cocci that not only were resistant to strepto mycin but even required it for growth The development of streptomycin dependence has since been observed among many bacterial species in addition, many resistant mutant strains are stimulated by streptomycin even though they do not have an absolute require ment for it There have also been a few reports of resistant mutants stimulated by penicillin, tetracycline and chloramphenicol

Drug dependence presents a remarkable metabolic problem A possible explanation has appeared following the discovery of a sulfanil amide requiring mutant of the mold, *Neuro-spora Zalokar found that this mutant is* inhibited by its endogenous PAB apparently not because of increased production of PAB, but rather because of abnormal sensitivity to inhibition by a product of PAB metabolism Sulfanilamide therefore acts as a growth factor by preventing the formation of toxic amounts of this product rather than by functioning as a nutrient

Clinical and Epidemiologic Importance of Drug Resistance and Dependence

The frequency of emergence of drug resist ant mutants in patients under treatment varies widely with bacterial species and with chemo therapeutic agent Streptomycin is the worst offender and the sulfonamide group the next while penicillin and the chloramphenicol tetracycline group are relatively free of this defect

Resistant strains are particularly apt to emerge during the prolonged treatment of a chronic disease in tuberculosis resistant or ganisms generally appear in large numbers after 1 to 3 months of treatment with strep tomycin This rate is not excessively slow when considered in terms of the number of genera tions since this organism has a mean genera tion time some 30 times longer than that of most bacteria

Patients under treatment with streptomycin have yielded not only resistant strains of bac teria but also strains that are stimulated by or even require this compound Therefore, to continue therapy with this drug after the de velopment of a large number of resistant organisms not only may be useless but in some cases may be a positive hazard However the problem is complex for the presence of re sistant tubercle bacilli in the sputum does not exclude the existence of foci of susceptible organisms as well

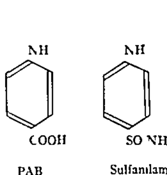
The problem of drug resistance has impor tant epidemiologic aspects Not only can re sistant strains emerge in a patient under treat ment but the widespread use of a drug among

advantage studied carefully by disinterested investigators

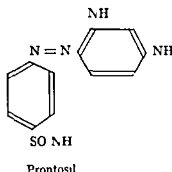
THE INDIVIDUAL CHEMOTHERAPEUTIC AGENTS

Sulfonamides, Sulfones and p-Aminosalicylic Acid

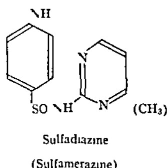
Following the discovery of sulfanilamide as the active breakdown product of Prontosil several thousand structurally related compounds were synthesized and tested. It was found that substitutions on the free amino group (N^4) including the acetylation that occurs in the body render the compound chemotherapeutically inactive but a variety of substitutions on the sulfonamide group (N^1) cause a marked increase in potency. Sulfanilamide itself was useful against few organisms except the streptococcus, the meningococcus and the gonococcus, but the more potent derivatives extended the useful antibacterial spectrum of sulfonamides to include the pneumococcus, the staphylococcus, dysentery ba



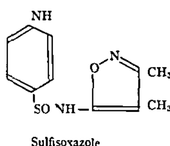
PAB   Sulfanilamide



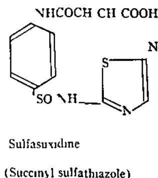
Prontosil



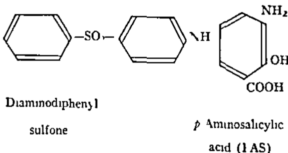
Sulfadiazine (Sulfamerazine)



Sulfisoxazole



Sulfasuxidine (Succinyl sulfathiazole)



Diaminodiphenyl sulfone

p-Aminosalicylic acid (PAS)

Unfortunately, though mutation rates can be increased by a variety of physical and chemical agents, no method is known for decreasing them effectively Therefore the best prophylactic approach to drug *resistance* appears at present to consist in attempting to suppress the *selection* of mutants since there is no way of suppressing their *formation* Along these lines the most promising approach is offered by combined therapy with two independently acting agents This solution, which was suggested by Ehrlich now has a clear rationale based on genetic principles if one cell in 10^6 mutates to resistance to one drug and one in 10^6 to another only one in 10^{12} will develop both mutations simultaneously Hence doubly resistant mutants have a negligible probability of emerging from a sensitive strain in the presence of effective concentrations of two chemotherapeutics with different *modes of action* even though such mutants can easily be obtained by selection first with one drug and then with the other The importance of combining drugs that lack cross resistance should be emphasized cross resistance is shown not only among the sulfonamides but also within the tetracycline group

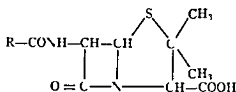
The principle of combined therapy has been applied clinically with encouraging results in treating tuberculosis with various combinations of streptomycin isoniazid and *p* amino salicylic acid Though doubly resistant strains do emerge eventually in some patients treated in this way this event is not in conflict with the genetic principle that underlies combined therapy for this principle requires that both *drugs be present in the patient at all times* during the period of therapy and this objective cannot be attained in a patient who is under treatment for months Hence in the treatment of tuberculosis combinations of drugs as used to date have prolonged the period of useful therapy but they have not completely solved the problem of drug resistance

Another advantage of combined therapy arises from the use of drugs with significantly *different distributions in the body* Thus penicillin and sulfonamides are often used together in the treatment of pneumococcal or meningococcal meningitis The rationale is that the penicillin, being bactericidal would eliminate more rapidly those organisms that it can reach while the sulfonamides, though only bacteriostatic, can penetrate much better into the central nervous system However meningococci respond so well to sulfonamides alone that the addition of penicillin may well have no real advantage in treating meningococcal meningitis

In this case, where the bacteriostatic agent alone could be adequate the added bactericidal drug might or might not help further but at least should do no harm In other cases where the bactericidal action of penicillin or streptomycin would be important the addition of a bacteriostatic drug could even suppress the bactericidal action by interfering with the metabolism required for that action Such interference has been observed both in vitro and in experimental animals for various pairs of drugs (Jawetz and Gunnison 1953) and clinically the addition of a tetracycline has been shown to decrease the effectiveness of penicillin in the treatment of pneumococcal pneumonia

An additional advantage of certain combinations may arise from the fact that variations in metabolic state cause some cells in a population to be less responsive to a drug than others In particular those cells that tend to remain viable in the presence of penicillin might well not be the ones that persist in the presence of streptomycin This consideration as well as differences in distribution may underlie the clinical observation that a combination of these two drugs has proved to be better than either alone in the treatment of enterococcal endocarditis Similarly, combinations of streptomycin with other drugs have been recommended for treating brucellosis though the tetracyclines alone now appear to be equally effective

While mixtures of chemotherapeutic agents may be valuable in special circumstances as described above one must deplore the general use of mixtures of chemotherapeutic agents as shotgun therapy to substitute for an accurate diagnosis Another justification for combined therapy that is being advocated is the hope of achieving synergistic (i e greater than additive) action Many pharmaceutical firms are now marketing a variety of prefabricated mixtures supported by claims of synergistic action This is a recent development and it would be desirable to have this alleged



Penicillin G R = [benzene ring]—CH

(benzyl)

Penicillin V R = [benzene ring]—OCH

(phenoxymethyl)

by Chain et al in 1939 The circumstances of its development during the course of World War II led to extensive governmental subsidy of penicillin production As a result it became possible to develop methods and facilities for the production of huge quantities of the drug within a year or two This development led to a new type of pharmaceutical production which was responsible for the rapid exploitation of other antibiotics soon after their discovery

The original strain of Penicillium yielded relatively small amounts of the antibiotic but it proved to be possible to induce formation of higher yield mutants by the use of ultraviolet irradiation By successive selection multiple step mutants with enormously increased yields have been obtained Furthermore it was formerly conventional to cultivate molds which are compulsory aerobes as a surface mycelial growth on shallow layers of medium but it has proved to be much more economical to produce penicillin by forced aeration of submerged cultures in tanks containing as much as 50 000 gallons of medium Through such developments the cost of producing pure penicillin has become minor compared with the cost of its packaging and distribution Similar procedures have been used to improve the yield of the other antibiotics Penicillin has also been synthesized chemically but the process cannot compete with the fermentation

The antibacterial substance in filtrates of the mold was designated originally as penicillin on the assumption that it was a single compound Subsequent purification revealed the presence of substantial quantities of at least 6 biologically active compounds which differed from each other only in the nature of the acid that was condensed in amide linkage with the bicyclic nucleus of the molecule (see p 688) Isotopic studies have shown that this nucleus which is common to all of the penicillins arises from the condensation of a molecule of D valine with one of D cysteine By the addition of various organic acids to the medium it is possible to force the production of a wide variety of other penicillins

The most suitable of the original penicillins with respect to yield and pharmacologic properties proved to be penicillin G (benzyl penicillin) and most of the penicillin marketed in recent years has consisted of various salts of crystalline penicillin G Penicillin V (see formula above) introduced more recently has become widely used for oral administration because it is more resistant to destruction by gastric acidity The various penicillins have identical antibacterial actions

The unit of penicillin (Oxford unit) originally defined in terms of the zone of inhibition of a standard strain of *Staph aureus* turned out to be equivalent to 0 6 μg of penicillin G This unit was introduced before pure material was available and it seems unfortunate that penicillin in contrast with the other antibiotics is still prescribed on the basis of an arbitrary rather than a gravimetric unit

The mode of action of penicillin has been discussed above It is rapidly bactericidal and lytic but only to growing bacteria Since it interferes with the synthesis of cell walls which exist in bacteria but not in mammalian cells its extremely selective toxicity is readily understood Unlike the sulfonamides it is not antagonized by pus or products of tissue autolysis

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cilli and the cholera vibrio, gram negative urinary tract infections, anthrax, chancroid and the large viruses of the trachoma lympho granuloma group However, sulfonamide action on the coccal infections is less immediate and less certain than that of various antibiotic chemotherapeutic agents that were discovered later These antibiotics may also be less toxic For these reasons they have largely displaced the sulfonamides for the coccal infections (except meningococcus meningitis) The sul fonamides are still used in specialized situ ations

While various sulfonamides differ in potency and hence in useful spectrum there is no selec tive affinity which makes one of these drugs particularly valuable against one organism and another against a different organism In other words sulfathiazole is over 100 times as potent as sulfanilamide against each bac terial strain Furthermore mutants with in creased resistance to one sulfonamide show a similar increase in resistance to all Hence the choice among potent sulfonamides is based not on specificity but on pharmacologic properties toxicity solubility and speed of elimination

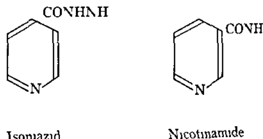
The sulfonamides still in use today in clude sulfisoxazole sulfamethoxypyridizine (Kymex), sulfadiazine and the closely related sulfamerazine and sulfamethazine (The fact that sulfamethazine is rapidly acetylated de creases greatly its usefulness) Furthermore while the N^4 substituted compounds are them selves inactive sulfathiazole substituted in this position with succinic acid (sulfasuxidine) or with phthalic acid (phthalylsulfathiazole) is useful as a *gastro intestinal antiseptic* Appar ently the N^4 substituent is slowly hydrolyzed in the gut providing a continuous supply of sulfathiazole

The mode of action of sulfonamides through competition with PAB has been described in an earlier section (p 676) where certain limi tations on the effectiveness of these drugs were also discussed (static and not cidal action delayed action antagonism by products of tis sue breakdown) An additional limitation arises from the fact that sulfonamides are less effective at low pH values and this property may be important in the treatment of urinary tract infections

Two other chemotherapeutically useful an alogues which compete with PAB have anti bacterial spectra quite different from those of the sulfonamides *Diaminodiphenyl sulfone* and its derivatives are the only chemothera p utic agents available for the treatment of leprosy in which they have real value even though they are not dramatically curative

Para aminosalicylic acid (PAS) is restricted in action at pharmacologically possible con centrations to tubercle bacilli Its effect is not as striking as that of isoniazid or strepto mycin, but it is useful in combination with either of these agents PAS must be used in unusually large doses

ISONIAZID



Isoniazid Nicotinamide

Isoniazid (isonicotinic hydrazide) has been known to organic chemists for decades Its value as a chemotherapeutic was discovered independently in 1951 in two pharmaceutical firms This discovery was particularly surpris ing since by that time chemotherapeutic re search had been largely shifted to antibiotics

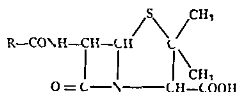
Isoniazid exerts a bactericidal action against the tubercle bacillus and it is effective in un usually low concentrations Its useful action is restricted to this disease in which it proved to be considerably more effective than the other available antituberculous agents (strep tomycin PAS) Because of the problem of drug resistance it is generally used in combina tion with one of these other drugs Its supe riority to streptomycin probably is related to the fact that it penetrates better into mam malian cells and hence appears to be more effective against intracellular tubercle bacilli

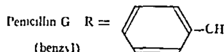
The mode of action of isoniazid is not known It has a structural resemblance to nicotinamide a component of di and tri phosphopyridine nucleotide (cozymase I and II) and enzymatic experiments have shown that it can be incorporated biosynthetically to yield an analogue of these coenzymes How ever it has not been established that this re action underlies its chemotherapeutic activity

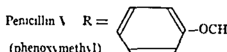
PENICILLINS

Penicillin is produced by certain strains of the molds *Penicillium notatum* and *Penicillium chrysogenum* which excrete it into the sur rounding medium

The existence of penicillin was discovered by Fleming in 1929 and it was partly purified and found to be a useful chemotherapeutic







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salt is used, administration of large doses can result in potassium intoxication in the presence of cardiac or renal insufficiency

The development of drug resistance during treatment is rarely a problem even in the case of nonhemolytic streptococcal endocarditis its occurrence is less frequent than was formerly believed However penicillin resistant strains of staphylococci are encountered frequently, particularly in hospitalized patients who presumably acquired the infection in the hospital These strains generally owe their resistance to the production of penicillinase an enzyme that hydrolyzes the unstable 4 membered (β lactam) ring In some organisms the production of this enzyme is constitutive but in others it is induced by growth in the presence of penicillin Its influence on the nature of the resistance has been discussed above (p 683)

The wide variations in the sensitivity of various strains of staphylococci to penicillin and to other chemotherapeutics present a special problem in therapy Therefore it is particularly valuable in staphylococcal infections to determine the pattern of sensitivity of the isolated organism

Antibiotics Resembling Penicillin in Antibacterial Spectrum

Erythromycin was discovered in 1952 in the filtrate of a strain of an actinomycete *Streptomyces erythreus* It consists of a 14 membered lactone ring containing a number of substituents including a novel aminohexose and a second unusual sugar Its mode of action is unknown It is bactericidal in vitro under optimal circumstances but it may also be only bacteriostatic under conditions where penicillin would be bactericidal Its spectrum is almost identical with that of penicillin It is used primarily for the treatment of penicillin resistant infections

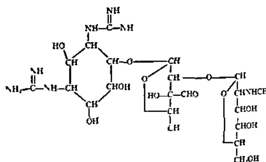
Carbomycin (Magnamycin) another product of an actinomycete has the same spectrum as erythromycin and shows complete cross resistance with it but is less effective Therefore it has little clinical use today

Oleandomycin (Matromycin) a product of an actinomycete has a similar spectrum and also appears to be less effective than penicillin or erythromycin

Novobiocin (Albamycin Cathomycin) is a complex coumarin derivative containing a phenolic group which is produced by *Streptomyces niveus* and *Streptomyces spheroides* It has a spectrum much like the preceding antibiotics but does not show cross resistance with them Therefore it is particularly valuable against penicillin resistant staphylococci The alleged value of novobiocin against some infections with *B proteus*—an organism usually unresponsive to chemotherapeutic agents—remains unproved

Bacitracin is a polypeptide produced by a strain of *Bacillus subtilis* first isolated by Meleney from a patient named Tracy Its spectrum is similar to that of penicillin Like other polypeptide antibiotics (gramicidin polymyxin) it is quite toxic and so it should be used systemically only for strains that are resistant to less toxic agents

STREPTOMYCIN



Streptomycin was discovered in 1944 by Schatz Bugie and Waksman as a product of the actinomycete *Streptomyces griseus* This was the first of a number of chemotherapeutic antibiotics obtained from actinomycetes It is an exceptionally highly polar compound consisting essentially of an inositol substituted with two guanido groups and a disaccharide It contains three basic groups which account for the fact that it precipitates nucleic acids in vitro

The basic groups are undoubtedly also involved in its antibacterial action since its effectiveness falls off rapidly with increasing salt concentration presumably through competition between cations This effect has practical importance for the treatment of urinary tract infections Its action is also strikingly antagonized by H^+ ions even in the limited range of pH (down to 5 5) of ordinary bacterial cultures or urine This effect of acidity undoubtedly contributes to the decrease in the effectiveness of streptomycin observed in caseous or purulent foci as well as in acidic urine

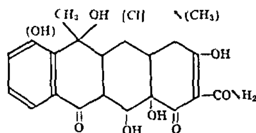
The bactericidal action of streptomycin requires metabolic activity (e g the presence of an energy source) but not necessarily growth Its mode of action is not clear and no metabolic antagonists are known

Streptomycin is active against certain of the gram positive organisms that are susceptible to penicillin but originally its particular importance lay in extending the range of useful chemotherapy to include the tubercle bacillus and many gram negative bacillary infections Because of its fairly marked toxicity it has been largely displaced by the broad spectrum antibiotics for use against gram negative organisms and it is inferior to penicillin in the treatment of coccal infections Therefore it is mainly used now in the treatment of tuberculosis and also in combined therapy for some of the more intractable chronic infections such as subacute bacterial endocarditis or brucellosis Because it is excreted in the urine mostly unchanged it is also useful in treating urinary tract infections caused by such organisms as *E coli* Aerobacter or Proteus

The value of streptomycin is limited not only by its toxicity but also by the fact that many organisms readily develop resistance to it

Reduction of the aldehyde group of streptomycin yields *dihydrostreptomycin* which has somewhat different toxicity but an identical antibacterial action

BROAD SPECTRUM ANTIBIOTICS
TETRACYCLINES



Tetracycline (Oxytetracycline)
(Chlortetracycline)

Chlortetracycline (Aureomycin) a product of the actinomycete *Streptomyces aureofaciens* was discovered by Duggar et al in a pharmaceutical laboratory (Lederle) in 1949 A short time later an identically acting antibiotic oxytetracycline (Terramycin) was isolated from a different Streptomyces species by a competing firm (Pfizer) It was shown to differ slightly in structure (see above) hence it could be patented as an independent composition of matter Later a third drug tetracycline itself was developed by chemical treatment of chlortetracycline and also (in the Bristol laboratories) as a direct product of still another Streptomyces The 3 tetracyclines have identically antibacterial actions and they exhibit essentially complete cross resistance Since tetracycline itself the last to be discovered is the most stable and hence the best absorbed on oral administration it seems to be destined to replace the other two despite commercial efforts to defend them

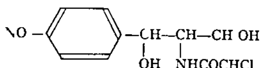
The mode of action of the tetracyclines is unknown It is generally regarded as bacteriostatic though a bactericidal effect can be seen under certain conditions

The tetracyclines have a broader spectrum than any of the antibiotics described above They are effective against most of the bacterial species (but less against the spirochetes and actinomycetes) that are sensitive to penicillin but because of their greater toxicity tetracyclines are generally not the preferred drug in patients with such infections (except for patients allergic to penicillin) Tetracyclines are also effective against most gram negative organisms and have largely displaced streptomycin in their treatment It should be noted however that neither agent is often effective against infections due to salmonella (including typhoid) proteus or pseudomonas Finally tetracyclines are dramatically effective against rickettsiae and they have proved to be useful against brucella the large viruses of the lymphogranuloma group and certain protozoa (amebae trichomonas) In the case of protozoa the action may depend on depriving the pathogen of the bacteria it uses as food

Investigators had long sought an antibiotic with the widest possible antimicrobial spectrum in the hope of increasing the number of curable infectious diseases and also eliminating failures due to a faulty (or absent) etiologic diagnosis With the discovery of the tetracyclines it appeared that this objective was being closely approached However it now turns out that the advantages of a very broad spectrum may be outweighed by the disadvantages In some patients the tetracyclines so completely eliminate the normal flora of the gut that there results after a sufficient period of therapy a considerable overgrowth of resistant staphylococci and occasionally yeasts (especially Monilia = *Candida albicans*) Frank monilial infections (vaginitis pneumonitis) also appear occasionally The abnormal flora of the gut has been considered largely responsible for the most frequent toxic effects of these drugs (enteritis perianal and oral in

flammation) However, since there is not a consistent parallelism between the appearance of such symptoms and the extent of demonstrable suppression of the normal flora it is quite possible that a direct toxic effect of the drug is the major source of difficulty In any case the suppression of normal flora is not consistent enough to make the tetracyclines a valuable intestinal antiseptic

CHLORAMPHENICOL



Chloramphenicol (Chloromycetin) was isolated by Burkholder et al from *Streptomyces venezuelae* This actinomycete was found in a sample of South American soil obtained during a trip to collect possible sources of antibiotics from various parts of the world Subsequently, the same organism was rediscovered in a soil sample from the grounds of the firm in Michigan that undertook the production of this antibiotic[1]

The structure of the drug is most unusual for a biologic product in that it includes a nitro group and a dichloracetyl group Though discovered as an antibiotic the drug is now produced commercially by chemical synthesis rather than by fermentation

Chloramphenicol is a bacteriostatic agent It has been shown (Gale Wisseman) to block protein synthesis in bacteria—an action that has proved to be useful in the study of protein synthesis Ordinarily the synthesis of protein goes hand in hand with the synthesis of ribonucleic acid any interference with the formation of one (e g deprival of a required building block) immediately prevents formation of the other However chloramphenicol allows the cell to continue to synthesize considerable RNA without protein

The antibacterial spectrum of chloramphenicol is similar to that of tetracycline It is often stated that the two also exhibit cross resistance However, this conclusion which would be surprising in view of their structures is questionable It is true that within what are considered generally susceptible species various strains that are resistant to both drugs are often found in nature but when one isolates in vitro a mutant that is selected for increased resistance to either drug the change is not usually associated with increased resistance to the other

Chloramphenicol is a much less frequent source of gastro intestinal toxicity than the tetracyclines This advantage may be related to the fact that it is better absorbed, and hence less reaches the lower bowel It is also more effective in the treatment of typhoid but neither drug is effective against other salmonellas or against the typhoid carrier state

Chloramphenical gives rise though rarely to aplastic anemia and other forms of depression of bone marrow activity Since these complications are sometimes fatal for some years the use of chloramphenical tended to be restricted to the treatment of typhoid fever and of meningitis caused by *Hemophilus influenza* However, because the dangerous complications are very rare, and because chloramphenicol has distinct advantages it is regaining general use as a broad spectrum antibiotic

ANTIFUNGAL AGENTS

Nystatin (Mycostatin) is a product of a Streptomyces Its name is derived from the fact that it was discovered in the laboratories of the Department of Health of N Y State It is active against yeast and molds and particularly against moniliasis It is often used along with tetracycline to prevent this complication of tetracycline therapy

Amphotericin B also a product of a Streptomyces is a recently introduced drug that is stated to be useful against a variety of fungal infections

MISCELLANEOUS ANTIBIOTICS

Polymyxins (Aerosporins) are a group of polypeptides produced by a bacterial species They have considerable renal toxicity and so are not used systematically for infections that respond to less toxic drugs However the least toxic member of this group polymyxin B is often useful against *Pseudomonas aeruginosa* (*B pyocyaneus*) which is usually unresponsive to other chemotherapeutics It is also used for gastro intestinal antisepsis

Neomycin a polybasic product of an actinomycete is particularly effective against gram negative rods It is so toxic that it is rarely used systemically However on oral administration it is absorbed only negligibly and so it is a highly effective preoperative intestinal antiseptic

Cycloserine is a rather simple compound excreted by an actinomycete

It is active against the tubercle bacillus but should be used only in refractory cases until more is known of its toxicity

Kanamycin a recently introduced polybasic product of an actinomycete resembles streptomycin and neomycin in structure It is claimed to be useful in the treatment of strains resistant to penicillin

For *topical therapy* certain relatively toxic antibiotics including tyrothricin (gramicidin plus tyrocidine) bacitracin neomycin and polymyxin are used in preference to the less toxic agents The major reason is that topical *application appears to increase the likelihood* of developing an allergic reaction and it would be undesirable to risk such a development with an agent such as penicillin streptomycin or tetracycline which the patient might later need for a life threatening illness

In closing it might be noted that the production of antibiotics in 1956 in the United States amounted to 2 500 000 pounds

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WALSH McDERMOTT

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Chemotherapy of Microbial Diseases

In general, there seems to be no reason to believe that the reactions between appropriate number of molecules of an antimicrobial drug and an individual bacterial cell are any different in vivo from what might be observable in vitro Our difficulties arise less from inadequacies of in vitro technics per se than from our inability to identify the precise conditions operative in vivo which should be imitated with these technics Drug microbe relationships within the body are considerably more complicated than in conventional in vitro systems because of the existence of three sets of circumstances

1 Unlike a solution of nonliving molecules a microbial population is a collection of biologic units each of which has a very considerable capacity to undergo physiologic changes in response to its environment In vitro this adaptive plasticity of the individual microbe is kept within fairly rigid boundaries because all of the microbes are existing in an essentially identical environment and usually are maintained there for only a short period of a few hours or days Within the body however microbes can survive for months or years and the host is capable of providing a wide range of environments Consequently a much greater opportunity exists for the microbe to display physiologic heterogeneity As will be discussed subsequently, various physiologic states of the microbe may be associated with decreased or increased susceptibilities to particular antimicrobial drugs In certain situations however the homeostatic mechanisms of the host serve likewise in the interests of the microbe thus permitting the latter to obtain a continuous supply of nutriment in a continuously stabilizing environment As a consequence the parasites in vivo are not invariably subjected to the deleterious effects of a progressively decaying environment as is the case in vitro

2 Another difference between conventional in vitro systems and conditions in vivo is the fact that the usual physiologic activities of the host may serve to destroy or to inactivate an appreciable portion of a drug or may hinder its distribution into areas of infection Likewise the inflammatory lesions of the disease process itself may also hinder free distribution of a drug or might provide a biochemical environment that either neutralized drug activity or favored microbial states of drug insusceptibility

3 Finally the host possesses a number of different types of mechanism for the inhibition or the destruction of microbes These mechanisms when operating in association with a drug at times may serve to enhance and at other times may limit its effectiveness Almost certainly a number of such defense mechanisms exist that are as yet unidentified Hence it is highly probable that only the merest beginning has been made in the direction of reproducing host defenses in vitro

The existence of these circumstances which can influence both drug and parasite within the body so substantially makes it imperative to regard the chemotherapy of infections in terms of a triangular interrelationship between drug parasite and host In the subsequent discussion the simultaneous operation of these three factors should be regarded as implicit However as host parasite relations are discussed elsewhere in this volume the principal emphasis in the sections which follow will be

placed on the other two components of the 3 way system

DRUG HOST RELATIONSHIPS

Physiologic Activities of Host

The amount of drug actually available for antimicrobial activity at the site of infection represents only that portion of the administered dose which has survived the many neutralizing mechanisms of the host

Destruction or Elimination

The fractions of the various drugs which are diverted from antimicrobial activity by destruction or elimination by the body have not been determined in precise detail but it is safe to say that they are great It is known for example that an appreciable portion of penicillin administered by the oral route is destroyed by the acid of the stomach Likewise on intramuscular injection a proportion of some drugs may be precipitated locally and hence not be available for absorption during the period of active therapy Certain drugs for example penicillin and chlortetracycline are well absorbed from only a definite segment of the gastro intestinal tract and once past that area are excreted in the feces

It is conceivable that a relatively weak antimicrobial drug might be compromised in its effectiveness if it were excreted through the kidneys at an unusually rapid rate However this phenomenon has not yet been observed because the most rapidly excreted compounds penicillin and chloramphenicol have such high activity and low acute toxicity that they can be administered in quantities sufficient to balance rapid excretion in the urine

Once a drug has been absorbed into the circulation a greater or lesser proportion of it may be converted into inactive form This may proceed chiefly by acetylation as with isoniazid by glucuronidation as with chloramphenicol or by both of these mechanisms as in the case with the sulfonamides The inactivation of many of the newer drugs of microbial origin within the body has not been defined precisely It is established however that with certain of these drugs e g chlortetracycline and oxytetracycline only a small portion of the drug excreted in the urine is in biologically active form It seems likely therefore that inactivation within the body occurs to some extent with most if not all antimicrobial drugs

Interference With Distribution

In general the antimicrobial drugs available today are freely distributed through the blood and the extracellular fluid as evidenced by their rapid appearance in thoracic lymph and in agar disks implanted within the peritoneal cavities of various animal species (Werner et al 1951) Certain of the drugs are considerably more effective against a particular microbial species when its members are situated extracellularly than when they are located within host cells Whether or not this represents an actual failure of transfer of the drug to the internal environment of the cells has not yet been established and indeed is probably not the ca e Alternative explanations exist and will be discussed subsequently From studies in man it has been demonstrated that antimicrobial drugs penetrate readily into the various body compartments such as pleural pericardial and joint cavities and are present there in concentrations equivalent to those in the plasma However a most important exception from such free distribution exists with several drugs with respect to the central nervous system or the chambers of the eye

Whatever may be the factors which determine the transfer of a drug from the general circulation into the central nervous system they are obviously very precise Shannon and his associates (1943) have shown for example that considerable differences in the amounts of drug transferred may exist among very closely related compounds such as the various sulfonamides It is generally believed but by no means unequivocally proved that the transfer of drugs from the plasma into the central nervous system is facilitated in the presence of inflammatory disease of brain and meninges It should al o be noted that in order to obtain antimicrobial activity in the fluids of the central nervous system comparable with that present in plasma it is not always necessary to attain so high a concentration of drug as in the plasma This is because of the low albumin content of these fluids in contrast with plasma Such a situation obviously obtains only if the particular drug in question is neutralized to a substantial degree by binding to protein

Protein "Binding"

One of the mechanisms by which antimicrobial drugs suffer diminished activity while in the body is by interaction with protein as noted above in connection with in vitro tests. The importance of the "binding" mechanism in neutralizing drug activity in the normal fluids of the host has been established by the studies of Davis (1943) and Tompsett et al (1947). However, it is also probable that "binding" mechanisms as yet undefined but of this general type may be of even greater importance in certain normal tissues or in areas of inflammation or necrosis. The latter aspect of the question will be mentioned subsequently; the present discussion is confined to the known "binding" mechanisms in normal fluid.

As the "binding" to protein, principally albumin, is virtually instantaneous, the mechanism becomes operative immediately after the drug is absorbed into the circulation. The binding is reversible and depends on a chemical equilibrium. The albumin acts as a sponge, so to speak, by which a variable and at times a substantial percentage of drug is removed from a situation in which it can exert antimicrobial activity. However, the "bound" drug can be released to replace unbound drug in the fluid environment as the concentration in the latter falls. The individual members of a particular series of antimicrobial compounds vary widely in the degree to which they are bound to albumin, and in some cases (penicillin K) the percentage bound may be as high as 90 per cent.

Failure of Host Mechanisms To Be Wholly Effective

By all of these host mechanisms, such as neutralization, impeded transfer, or protein binding, a variable and usually quite large proportion of an administered antimicrobial drug is directed away from access to the parasite. However, none of the mechanisms cited is wholly effective and some drug, usually a small portion of the administered dose, does manage to elude the host mechanisms and achieve contact with the parasite. The question thus arises as to how much drug is needed to be effective at the site of infection. More precisely, the question is how many molecules of a particular drug are necessary to enter into a maximal reaction with an individual bacterial cell. Information on this point is scanty, but it appears that the quantities involved may be quite small, especially when considered in terms of the conventionally administered doses. In the case of penicillin, by isotopic labeling it has been estimated that approximately 1,000 molecules of the material can enter into the metabolic reactions of an individual parasite (Cooper, W., 1956). This would represent only an infinitesimal portion of the penicillin concentrations usually present in the body fluids of a patient receiving the drug. The factor of time also enters into the question because certain of the host mechanisms for drug neutralization, notably acetylation, are presumably slower than drug-microbe reactions. This subject of time-dose relationships in antimicrobial chemotherapy is presented in a subsequent section. At this point the fact of importance is that the drug neutralization mechanisms of the host, although obviously impressive, do not really provide satisfactory explanations of why certain chemicals active in vitro are apparently inactive in vivo. Consequently, it seems likely that as yet undisclosed mechanisms must be sought to explain the familiar phenomenon whereby a compound exerts definite antimicrobial activity in vitro but fails to protect animals infected by the corresponding parasite.

DRUG-PARASITE RELATIONSHIPS IN VIVO

Perhaps the best point of departure for consideration of the mechanics of drug-parasite relationships in vivo is a scrutiny of the phenomenon of relapse. In any group of patients with a particular infection, the administration of an antimicrobial drug appropriate for the agent of that infection is followed by the remission of all evidences of illness. With a few infections and with certain drugs, the illness will reappear eventually despite continued drug therapy, and this reappearance will be associated with the emergence of drug-resistant strains of micro-organisms. In most situations, however, the administration of an appropriate drug results in remission of all evidences of illness for an interval which continues beyond the period of drug administration. If the period of drug therapy is of sufficient length, a factor that varies considerably among different in-

fections the remission will be permanent in the majority of patients However with virtually all infections and all antimicrobial drugs in certain persons not otherwise distinguishable from the rest a period of antimicrobial therapy sufficient for the majority will be insufficient In this minority the asymptomatic post treatment interval will be sharply terminated by a return of the manifestations of infection Finally there is a third pattern observed only rarely in which the remission of the original infection is followed immediately by infection of the same organ or system by a microbe of a different species

It is obviously of the greatest importance to determine whether relapsing illnesses caused by the same microbial species primarily represent a failure to complete the particular drug parasite reactions known from in vitro studies or whether they reflect the existence of other types of drug parasite relationships which can now only be surmised from in vitro studies In other words does the phenomena of relapse represent mere quantitative failures in carrying a single type of drug parasite reaction to completion in *all* of the infecting population or is there a variety of possible types of drug parasite contacts only some of which lead inevitably to the death of the parasite

On superficial examination the form of relapse that occurs in association with the emergence of drug resistant microbial strains might seem to be not intimately related to the common form of post treatment relapse On more careful scrutiny however it is clear that this is not wholly true and that factors having to do with relapse in general also have bearing on the less frequently encountered forms of relapse associated with drug resistant microbes As a consequence discussion of the latter form will be deferred until after consideration of the more common form of relapse in which drug resistance in the orthodox sense of the term does not seem to be a factor

It is well recognized that the clinically successful treatment of a particular infection may be followed by a post treatment carrier state with respect to the offending microbe and yet this state does not necessarily lead to relapse Thus it is clear that there is more to relapse than merely the continued presence of the microbe in the tissues of the host although the continued presence of the microbe is obviously essential The basic question is whether by any practicable manipulations it is possible to eradicate an infection *with uniformity* by drug therapy or whether the final elimination of all of the microbes can be accomplished only by host parasite mechanisms over which the therapist has no real control

There has been a tendency to become unduly preoccupied with the question of whether a particular drug in its customary relationships with a parasite is bactericidal or bacteriostatic Such information however valuable in helping to define the nature of the reaction between a particular drug and a particular microbial species is really only a minor aspect of the much larger question of whether a drug is truly eradicative or is merely suppressive

The clinician who sees relapse following what should have been adequate therapy with a so-called bacteriocidal drug is much more impressed with the failure of drugs to be eradicative than is the laboratory investigator who studies these problems only in the test tube or in host species other than man Indeed on the basis of a number of clinical observations reinforced by pointed experiments in the laboratory it is quite clear that the antimicrobial drugs available today are predictably eradicative only in the case of a few microbial species Considerable differences exist among the various species with respect to the speed with which the microbe autolyses following interference with an important metabolic process It is particularly pertinent to note therefore that the few situations in which antimicrobial drugs appear to be eradicative involve two of the most fragile species meningococci and dysentery bacilli It is further worthy of note that the two drugs involved in the eradication of these species from the host the sulfonamides and the tetracyclines are both classified as bacteriostatic on the basis of in vitro testing against other microbial species In short the determining factor in eradication appears to be the relative fragility of the particular microbial species and not the type or the degree of the antimicrobial activity exerted by the drug In this connection the possibility must also be considered that successful chemoprophylaxis of infection as distinguished from the early treatment of disease may prove to be limited to these few microbial species with the most

marked tendency to die once they are inhibited by a drug

The previously mentioned clinical observations on the failure of drugs to be predictably eradicative had to do with attempts to prevent a beginning infection from becoming disease to abolish *post treatment relapse and* to eliminate the natural or post treatment carrier state The attempts to prevent infection from becoming disease will be discussed subsequently in the section on the influence of antimicrobial therapy on immunity Suffice it to say at this point that in all 5 diseases for which observations are available (malaria, scrub typhus Q fever syphilis and tuberculosis) the introduction of powerful antimicrobial therapy even in the early hours of infection does not lead to the eradication of the parasites from the host The attempts to abolish relapse and the postinfection carrier *state have followed two approaches of increasing* the size of the daily dosage of drug and extending the total period of antimicrobial therapy

The first approach (increased daily dosage) has been used in an attempt to reduce the incidence of post treatment relapse in two penicillin susceptible infections—syphilis and the bacterial endocarditis caused by viridans type nonhemolytic streptococci However even the daily administration of 10 to 30 million units of penicillin for periods of 10 to 14 days did not appreciably lower the incidence of relapse in these two infections (Bundesen 1947 King 1951)

The second approach extending the period of therapy has been associated with a substantial reduction but not the abolition of both relapse and the carrier state after therapy *With the carrier state it is possible by the* use of chemotherapy to produce a situation in which the offending micro organisms cannot be cultured during or shortly after a course of the therapy However in a portion of those treated (size of *portion depends to a certain ex*tent on length of therapy) the micro organism in question will either persist throughout therapy or reappear soon after it is stopped The results of therapy of the established carrier state produced by various micro organisms are similar to those observed in attempts to prevent the postinfection carrier state

All known strains of pneumococci are extraordinarily susceptible to the action of penicillin both in vitro and in vivo The action is exerted so rapidly in vivo that the first evidence of the halting of the onward progress of pneumococcal pneumonia may be perceived within 12 to 24 hours of the start of penicillin therapy *In virtually all instances if the peni*cillin therapy be continued for a total period of 10 days, relapse is not to be anticipated thereafter Despite this unquestionably powerful antipneumococcal action of penicillin in pneumonia it is not uniformly possible to eliminate a particular type of pneumococcus from the nasopharynx of healthy carriers by the administration of penicillin for a comparable period of 7 to 10 days To be sure the micro organisms may not be demonstrable on culture during the period of chemotherapy, but soon after its completion they reappear

Other examples of the failure of antimicro*bial drug to be uniformly effective in eliminat*ing a "carrier state may be seen in the case of chloramphenicol and *S typhosa* and penicillin and Group A streptococci In fact with the exception of meningococci and the sulfonamides and dysentery bacilli and the tetracyclines there probably are no instances in which the carrier state of any host parasite relationship can be eliminated uniformly by introduction of an antimicrobial drug

In the natural recovery from infections such as pneumococcal pneumonia or streptococcal pharyngitis the offending micro organisms eventually disappear from the nasopharynx in the majority of persons Therefore it may be inferred that mechanisms exist whereby the carrier state may be eliminated by the host In nasopharyngeal 'carriers" of Group A streptococci if the period of penicil*lin therapy be sufficiently prolonged* unquestionably the incidence of the 'carrier' state can be diminished markedly However in view of the failures of short term therapy (e g 7 days) in this situation it is difficult to judge *whether the eventual eradication of* the parasites on long continued therapy represents a direct consequence of drug action or is merely the result of either the natural death of the parasites or the eventual operation of influ*ences of the host Nevertheless it is conceiva*ble that with very drastic prolongation of therapy of the order of 6 weeks of penicillin for streptococcal pharyngitis and 6 to 12

months of penicillin for syphilis or of tetra cycline for brucellosis it might be possible to abolish completely both relapse and post treatment carrier' state However such a course would necessitate an impracticable pro longation of therapy for the majority in order to attain the desired result in the minority

In actual practice therefore the chemother apy of an infection consists of two steps (1) an abrupt halting of the onward progress of the infection with a consequent abolition of clinical illness (2) administration of drug therapy thereafter for a period empirically determined to be associated with a tolerably low incidence of relapse It should be recog nized that in these circumstances all of the microbes causing a particular infection are not necessarily eradicated during chemotherapy and that the disposal of the survivors depends upon the host or the natural death of the parasites

Therefore as they are currently used the available antimicrobial drugs are seldom uni formly eradicative whether they ever could become so remains open to question The prin cipal obstacle in the way of settling this ques tion is the fact that it is so difficult to obtain specimens from a lesion of a human at the periodic intervals necessary for proper quanti tative study Investigation of micro organisms remotely *derived* from lesions such as bacteria in the sputum from a patient with pulmonary disease or of micro organisms existing in the nasopharynx of a healthy carrier provides information which is important but does not necessarily bear directly on the question Moreover studies in animals are subject to the defect that the result of a particular host parasite interreaction can be different among different host species

An example of the last named type of situ ation is provided by the experiences with the penicillin treatment of syphilis in rabbits and in man which are discussed in detail elsewhere in this volume *Treponema pallidum* is among the more susceptible of micro-organisms to penicillin The nature of the action of the drug in this situation has been described as bactericidal In the rabbit the time do e relationships of penicillin therapy which will be associated with complete disappearance of *T pallidum* can be calculated with mathe matical precision Nevertheless when the identical drug parasite situation is present in man the eradicative dose of penicillin has not been found despite systematic trial of a wide variety of regimens including the feasible extremes of time dose relationships

In some 10 to 15 per cent of the infected persons who receive penicillin treatment, a return of the overt manifestations of the in fection occurs within a few months to a year of the first illness Certain of these second at tacks undoubtedly represent reinfections but there is every reason to believe that an impor tant percentage are true relapses There is no reason to assume that penicillin is any less active within man than it is in the rabbit but the end results of the drug parasite host inter actions are considerably different in the two situations In the rabbit penicillin exerts an apparently uniformly eradicative effect against *T pallidum* in man it is not uniformly eradicative

Despite the fact that identical drug parasite relationships may lead to different outcomes in different host species much information has been derived from studies of the fate of mi crobes in the tissues of drug treated animals The phenomenon of microbial persi tence i e the capacity of microbes to survive drug exposure in the tissues despite susceptibility to the drug in vitro has been demonstrated and studied extensively with staphylococci tuber cle bacilli and to some extent with Group A streptococci (McDermott W 1958) It has also been demonstrated that the same drug microbe pairings lead to considerably different outcomes in the different organs and tissues of the same animal Moreover it has been demonstrated that with certain drugs popu lations of tubercle bacilli under the influence of one drug in the tissues may behave in a distinctly different way to a second drug than wh n the latter is used alone Finally in this connection it has been possible to render cer tain microbial infections dormant and one infection (*M tuberculosis*) truly latent * in the tissues of appropriately treated animals (McCune R et al 1956)

* By dormant is meant an infection in which the microbial population has stabilized in the ti ues at a low but detectable le el whereas the term latent designates an infection in which the microbes are undetectable by any available method except as they reappear at the time of relap e

What then are the explanations for the failure to carry a drug parasite reaction to completion with uniformity in a group of patients with a particular infection? Let us assume in the first place that we are considering only those situations in which the particular drug in question has been administered in large doses for a minimum period of 7 to 10 days Four general types of explanation have been offered

(1) That *the micro organisms which* survive represent the more drug resistant fraction of the total infecting population—the most resistant members of the phenotype so to speak (2) that certain of the parasites are so situated somewhere within the body that they are not reached by the antimicrobial drug (3) that the biochemical nature of the inflammatory necrotic environment neutralizes drug activity even though it does not impede its free distribution throughout the lesion and (4) that certain members of the microbial population are subsisting at such a low level of metabolic activity throughout the entire period of chemotherapy that they are physiologically insusceptible to the drug

Least Susceptible Fraction (Phenotypic Resistance)

It can readily be demonstrated in vitro that the individual members of a bacterial population show some variation in their respective susceptibilities to an antimicrobial drug comparable differences presumably occur in vivo In other words there is usually some variation in drug susceptibility within the phenotype The notion that these slightly less susceptible bacterial *cells survive chemotherapy and give* rise to relapse is hardly tenable when it is considered that such relatively small differences should be easily surmountable in view of the wide range of dosage possible with the available antimicrobial drugs It is also unlikely that the microbial survival depends principally upon the birth of genotypic drug resistant variants which then fail to emerge to predominance but instead persist for long periods as a minute beachhead for eventual relapse This point is a complicated one as will be discussed subsequently Nevertheless it can be said that in the absence of other factors tending to hold an infection dormant or latent drug resistant *variants characteristically* emerge to predominance in a drug containing environment Indeed this may easily be observed in certain specialized conditions notably streptomycin therapy There is convincing evidence, however, that the usual post treatment relapse such as may occur for example in streptococcal or pneumococcal disease is not produced by this mechanism

Inaccessible Microbes

A possible explanation for failure of drug microbe contact in the tissues is that the microbes are situated behind some type of barrier This possibility is still frequently mentioned in the literature and the barriers are presumed to exist in the form of "fibrotic walls" of abscesses, large avascular areas certain body compartments or the outer walls of phagocytic cells To anticipate the argument that follows, it does not appear that any of these alleged "barriers do in fact prevent the actual *delivery* of the drug to the microbe in short, contact is probably not prevented Without question, however, the ultimate outcome of the drug microbe collision in certain of these circumstances may be considerably different than the outcome following a meeting of the same drug and the same microbial species in another milieu

Fibrotic Abscess or Cavity Wall Fibrin Barrier

Surprisingly few experimental investigations have been directed to the distribution of antimicrobial drugs through fibrotic abscess or cavity walls or through fibrin barriers surrounding the lesions In considering this question *it is essential to distinguish between the* problem presented by a fibrotic or fibrinous barrier around a lesion and the extent to which any substance can diffuse through a large area of completely necrotic tissue

Although the wall of a chronic abscess or a tuberculous cavity may contain very large amounts of fibrous tissue it is by no means avascular On the contrary like other living tissue it has a well defined capillary supply As the fibrotic wall is nourished by the extracellular fluid which forms the chief vehicle for these drugs there is no reason to believe that the wall hinders their distribution For the same reason a cellular tubercle with*out a significant necrotic* component cannot be considered to be aloof from the extracel

lular fluid and the substances it contains Indeed in tudies with intravenously admin istered dyes in animals the central areas of dense pulmonary tubercles have been well stained Moreover agar disks surrounded by a dense fibrous membrane after 4 weeks of residence in the peritoneal cavity of rabbits are freely penetrated by intravenously admin istered penicillin or streptomycin Studies of the contents of tuberculous cavities and of noncavernous tuberculous pulmonary lesions in resected specimens have revealed the pres ence of substantial concentrations of isoniazid or streptomycin within the lesions To the extent that the question has been studied therefore no evidence has been found to support the concept that a drug freely diffusible through the extracellular fluid has been pre vented from passing through a fibrotic wall

The situation with respect to fibrin as a barrier to drug distribution is less clear and the various types of arrangement of fibrin within or around lesions have not been de fined It is well established however that penicillin diffuses freely throughout fibrin clots in vitro It has also been shown that penicillin and streptomycin diffuse freely into agar di ks implanted within the peritoneal cavity of animals despite the fact that the disks are surrounded by a thick fibrin mem brane Thus with at least two drugs fibrin arranged either in bundles or as a surrounding membrane has not constituted a barrier to diffusion It is known however that fibrin membranes roughly comparable with those present in certain lesions (e g staphylococ cal) may act as a differential filter for albu min and globulin Therefore it is conceivable that the distribution of some drugs as yet unidentified might be impeded by a fibrin membrane

Necrotic Areas

The possible role of an area of necrotic tis- sue as an impediment to drug diffusion is quite another matter from the question of fibrotic or fibrin barriers An area of necrosis is the only truly avascular lesion and is remote from the nearest source of fresh extracellular fluid in proportion to its size In a rapidly necrotizing process such as klebsiella pneumonia it ap pears unlikely that substantial quantities of drug would diffuse through any but the most peripheral regions before slough occurred Contrariwise in the chemotherapy of a less fulminating process such as a staphylococcal abscess or an area of tuberculous caseation, drug is being continually delivered through the capillaries at the borders of the lesion for a period of months Diffusion gradients for compounds vary considerably depending upon the properties of the compound and the sub- stance through which diffusion takes place Nevertheless if the area of necrosis is fairly stable as is the case with some caseous foci and if the drug is also stable in that environ ment it is reasonable to assume that in time the drug which is being continually delivered to the outskirts of the necrotic area will even tually diffuse throughout its entire extent From such isolated observations as are avail able notably those with streptomycin and isoniazid in caseated areas it appears that this assumption is correct

Body Compartments

The subject of the distribution of drugs into body compartments was discussed previously It is only necessary to state at this point that the only body compartments which are not freely penetrated by all of the available anti microbial drugs—the central nervous system and the ocular chambers—are not usually the obvious place of origin of the relapse of an infection when relapse occurs

Intracellular Location

The extent to which an intracellular loca tion of a microbe may protect it from contact with a drug is not fully known A microbe re siding within a host cell presumably derives its nutriment from the same source as the latter namely the extracellular fluid a medium in which high concentrations of drug are easily attainable However it is conceivable that a selective barrier might exist at the cell bound ary which would impede or prevent the free transfer of drug into the cell from this sur rounding medium Indeed such a mechanism might represent one of the reasons for the failure of certain compounds to exert anti microbial activity within the body even though they are present in the plasma and excreted in the urine in biologically active form It is further conceivable that the various types of host cells which provide residence for microbes

(granulocytes, monocytes plasma cells, vascular endothelium, Kupfer cells, and other sessile phagocytes) might show considerable differences in their relative penetrability by a particular drug present in the extracellular fluid Even were penetration of one or all of these cell types accomplished easily, it is conceivable that the biochemical environment within the cell might be unfavorable for the maximal antimicrobial activity of a particular drug

A priori therefore there is an eminently reasonable case to support the concept that intracellular residence might provide a privileged sanctuary for the microbe wherein it would not be reached by inhibitory molar concentrations of a drug In addition, there are a number of observations on this subject that have been made in tissue cultures using various drugs and various microbial species All of these studies (Magoffin and Spink 1951 Mackaness 1952 Suter 1952 Tompsett, 1956) show essentially the same thing namely that certain drugs when present in the extracellular environment are less effective against certain microbes when the latter are situated within cells than against homologous microbes subsisting in the extracellular fluid Certain other drugs notably the tetracyclines and isoniazid appeared to be equally effective against the microbial species tested irrespective of the *factor of intracellular or extracellular residence*

The reason the matter cannot be regarded as settled is the fact that except for studies of Eagle (1954) with isotopically labeled penicillin the technics available do not permit measurement of the actual transfer of drug into cell Instead all that has been measured is the degree of drug effect on a particular microbial species subsisting within the cell Thus the possibility cannot be excluded that effective molar concentrations of drug are being delivered to the cell interior but that the environment there is unfavorable either for the microbe to become inhibited or for the drug to exert activity Indeed there are certain paradoxes that suggest the importance of microbial factors as opposed to the cell wall acting as an impediment For example in the tissue culture studies streptomycin is considerably less effective against *M tuberculosis* residing within cells than when the microbes are outside the cells Yet the same drug streptomycin, is highly effective in man against tularemia an infection that is usually considered to be predominantly intracellular in character To be sure, it is conceivable that in tularemia, the drug action is limited to the *parasites which* are existing extracellularly, such as those in transit from one cellular residence to another However if this should be the case it would follow from the rapid disappearance of clinical illness with drug therapy that the illness itself is largely produced by the small proportion of parasites situated outside host cells

A much more convincing body of evidence bearing on the question is provided by a comparison of the penicillin studies conducted respectively by Tompsett (1956) and by Eagle (1954) The Tompsett studies consisted of measuring in vitro the effects of various plasma concentrations of penicillin on staphylococci situated outside and within polymorphonuclear leukocytes suspended in the plasma As had been noted in previous studies with certain other drugs and other microbial species the effectiveness of the penicillin on the staphylococci was substantially lessened when the microbes were situated within the phagocytes Only 0 01 per cent of the staphylococci in the extracellular environment survived the first 24 hours whereas 10 per cent were able to survive within the cells

Despite this considerable reduction in penicillin effectiveness intracellularly nevertheless the populations of staphylococci within the *cells were reduced by approximately* 90 per cent Moreover in the Eagle studies with isotopically labeled penicillin and HeLa cells or mouse fibroblasts approximately 60 per cent of the penicillin present in the extracellular environment was actually transferred into the cells Therefore it was conceivable that the reduction of penicillin effectiveness noted by Tompsett was a consequence of too low dosage Accordingly he repeated the experiments using much greater concentrations of penicillin, namely 100 units per ml Even in this situation however from 5 to 10 per cent of the staphylococci survived when they were situated intracellularly Thus intracellular staphylococcal survival was not affected materially by an increase in extracellular penicillin concentrations well above those necessary for the inhibition of the staphylococci In this connection it is obviously necessary to make the assumption that HeLa cells and granulocytes behave in the same way in vitro with respect to penicillin transfer Such an assumption is not unreasonable however be

cause the similar drug microbe pairings that have been studied in more than one type of cell e g monocytes and HeLa cells have yielded similar results

Obviously it cannot be concluded from these two studies that cell boundaries are necessarily without influence on the transfer of drugs to the cell interior However what is strongly indicated is that in the one situation which permitted actual measurement of drug transfer the survival of the microbe intracellularly was not a consequence of a failure in drug delivery but depended on some other factors In short the interior of the phagocyte does not seem to provide a sanctuary wherein the microbe can remain *inaccessible* to antimicrobial drugs The persistence of the microbes within the single cell of the phagocyte appears then to represent a miniature replica of the situation of microbial persistence as it obtains in the host as a whole and not be significantly related to question of drug distribution

The third of the possible explanations of microbial persistence listed above is that the biochemical nature of the inflammatory necrotic environment is such that it neutralizes drug activity even though there is no significant impediment to the delivery of drug throughout the lesion This is an attractive concept and in the preceding edition of this text the present writer laid considerable emphasis on it as a possibility However on the basis of further consideration and new observations it no longer appears significant on critical scrutiny

The case for environmental antagonism of drug activity on a biochemical basis consists of the following facts that binding to macromolecular protein complexes might easily result in deviation of the drug from its appointed task of getting to the parasite that changes in the pH of the environment have marked effects on the antimicrobial activity of certain drugs and the pH of the inflammatory and the intracellular environments may be far from neutrality and finally that both tubercle bacilli and staphylococci attain considerably higher populations in some organs than in others

The obvious objection to the assumption that drug effectiveness is seriously impaired by macromolecular protein binding has been mentioned in a preceding section Such protein binding characteristically is an easily reversible phenomenon and the degree of binding depends upon the concentration of drug in the immediate environment Consequently even if macromolecular binding were occurring quantities of drug should also be released depending on the concentration in the surrounding environment When dealing with the large excesses of drug given for the prolonged periods customary in the treatment of a clinical infection it would seem that however active macromolecular binding might b it still would not sop up all the drug with which the lesions should be saturated

The factors of environmental pH and the different behavior of microbes in different organs of the same animal lend themselves to consideration together The observations that certain microbes are more susceptible to certain drugs when subsisting in one organ than in another obviously could represent environmental antagonism of drug activity An important objection to this interpretation arises from the observations mentioned previously of the behavior of staphylococci and of tubercle bacilli in the tissues of mice Both tubercle bacilli and staphylococci are usually inhibited in vitro by comparably small amounts of streptomycin In the case of the mouse spleen tubercle bacilli actually increase in numbers during the early days of streptomycin therapy and never fall below the pretreatment census when chemotherapy is given at the start of the infection In contrast with staphylococci *in the spleen* even in the absence of drug therapy the populations fall steadily and with streptomycin therapy the population of staphylococci fall to extremely low level Thus the same drug—streptomycin—which in effect is highly active in the spleen when it is acting on staphylococci is considerably less active *in the spleen* when it is acting on tubercle bacilli It is highly implausible that this relative antagonism of streptomycin action with tubercle bacilli represents an influence of the splenic environment per se on the drug It seems much more reasonable to infer that there is something about staphylococci or tubercle bacilli when they are subsisting in the spleen that make them show a difference in their reaction to streptomycin

In humans likewise there is a counterpart for this organ difference' For example penicillin is an excellent drug for the treatment of pneumococcal infection in the lung but is considerably less effective against the same microbe in the pleural cavity or the central nervous system The same type of statement would also hold true for streptomycin and tubercle bacilli namely that the drug is highly effective in the lung but considerably less effective in the pleural cavity or the central nervous system

In the same way that a drug acting in the same organ may be highly effective against one species of drug susceptible microbes while less effective against another drug susceptible species the same drug may exhibit widely different actions in different environmental circumstances in vitro For example it is well recognized that streptomycin loses a considerable portion of its effectiveness in partially *anaerobic environments This can be readily* demonstrated for staphylococci and for tubercle bacilli In identical circumstances however streptomycin is active against *E coli* (Mitchison et al 1956) Likewise pyrazinamide is inactive on tubercle bacilli of human origin when tested in environments within the physiologic range of pH Yet when the same environment is made acid the pyrazinamide is highly active on tubercle bacilli of human origin but not on bovine strains (McDermott et al 1956)

Thus it is easy to demonstrate that a change in environment may result in a substantial change in drug effectiveness However the changes in drug effectiveness that have been observed could represent as well the result of an environmental influence on the microbe rather than on the drug Moreover some of the observed changes appear to represent influences which could act only on the microbe The observations are to be further considered against the background of evidence that superfluous amounts of drug are usually well distributed throughout the lesions and presumably are also transferred to the phagocyte interiors In view of all of these considerations it seems likely that although environmental antagonism of drug activity may well occur it seldom if ever should attain sufficient magnitude to explain the ability of drug susceptible microbes to survive drug treatment in vivo and form the basis for subsequent relapse

If the tissue environment exerts no significant antagonistic influence directly on antimicrobial drugs *it must exert an influence on* the parasite For it is quite clear that in large measure the local environment determines the ultimate outcome of the drug microbe reaction Scrutiny of this question immediately leads to consideration of the last of the four suggested explanations for the phenomenon of microbial persistence and post treatment relapse This concept is that in all but a few microbial diseases certain members of the infecting population assume a state in which the particular metabolic reactions that are usually blocked by drug activity are not operating hence the microbes are invulnerable to the drug Thus it is presupposed that a microbe is capable of assuming a state in which it is *neither permanently incapacitated by a drug* nor does it multiply freely in its presence as do the genotypically drug resistant microbes In effect, therefore the microbe could be said to be "indifferent" to the drug and the presumed microbial state is designated drug indifference'

In considering this question it is necessary to presuppose that the nature of the environment can produce detectable changes in the parasite and that some of these changes affect its drug susceptibility Highly credible evidence can be assembled in support of these presuppositions Examples of the influence of the tissue environment in producing changes in the parasite have long been recognized notably in the emergence of bacilli from spores when the oxygen tension of the tissue is reduced In the case of other bacteria R G Wittler has observed a considerable morphologic change in *H pertussis* during a sojourn in the mouse lung More recently *Wittler and her associates (1956)* have observed the reversion of a pleuropneumonialike organism (PPLO) to a corynebacterium during residence in HeLa cell tissue cultures When in the pleuropneumonialike state the parasite showed little if any cytotoxicity for the cells in which it was situated until a yeast extract or a staphylococcal filtrate was added to the extracellular environment With the addition of either of these extracts considerable destruction of the "host tissues ensued and the pleuropneumonialike form was converted to the L form Subsequently the L form

was converted to the orthodox corynebacterium on further cultivation with the aid of mucin

Another example of tissue environment apparently determining a particular state of a parasite was demonstrated recently by Dubos (1957) in studies with *M fortuitum* This mycobacterium like tubercle bacilli or staphylococci proliferates to a much greater extent in certain tissues of the mouse than in other tissues of the same animal and the peak census occurs in the kidney Cultures of *M fortuitum* assume either one of two quite distinct forms in one form the colonies appear as a hard heaped up button without a peripheral lacy skirt in the other form the colonies are flat and extend for a considerable distance from the central core forming a peripheral skirt The important observation was that the microbial populations cultivated from one organ of the mouse assume one of the e colonial patterns whereas when cultivated *on identical medium* but from another organ the microbes assume the other colonial pattern It was further demonstrated that a purified substance known to be present in the tissues when incorporated into the medium could determine which of the forms was assumed by the parasite Therefore there seems to be every reason to believe that depending upon certain aspects of the environment in the particular organ the assumption of one or the other of these colonial patterns becomes predominant Thus the environment of the organ appears to determine the state of this particular microbe

The other presupposition that microbes are capable of assuming a state of drug indifference which is reversible can be very easily demonstrated by a simple experiment with penicillin and group A beta hemolytic streptococci in a favorable medium maintained first at icebox temperature and sub equently at incubator temperature In the cold environment the microbial population remains absolutely unchanged even though it is afforded a favorable medium and high concentrations of penicillin are present When the cultures are transferred to a warm environment two things happen simultaneously the population increases rapidly in the absence of penicillin and falls rapidly in the presence of penicillin This simple experiment which has been performed with a number of microbial species and a number of drugs clearly shows that there is a state of ' drug indifference that it is reversible depending on environmental changes and that such states can be induced or favored by the nature of the environment

To be sure the particular environments employed in the demonstration cited are highly artificial ones Nevertheless the same phenomena have b en demonstrated in conditions that more clearly resemble the conditions obtaining in the tissues during an infection

In general the published reports of the dynamics of the action of penicillin or other drugs in vitro have employed an essentially standardized system in which relatively small populations of microbes (10^6) are exposed to the drug With penicillin for example in these experimental systems there is a rapid and essentially straight line fall (on a logarithmic scale) of the microbial populations to low levels or indeed in many cases apparently to zero within a few hours It is the fact that this does not happen in the animal body that constitutes the phenomenon of microbial persistence

It has been shown that substantially larger populations (10^9) of streptococci are appreciably less responsive to penicillin in vitro than the smaller populations customarily employed in vitro (Bernsten C 1954) The large populations on exposure to penicillin in vitro show only a slow decline over time periods as much as a week or longer even though the strains are susceptible to penicillin on conventional testing It has been further shown that these large populations are comparable in size with those actually present in le ions in humans and in experimentally infected animals Moreover when these large populations are implanted in the muscle of a mouse which then receives penicillin the microbial populations display the same pattern of poor re ponse to penicillin observed with comparably large populations in vitro In large measure they appear to be drug indifferent

The question immediately arises in what ways other than mere size do these large populations of streptococci (which display drug indifference) differ from the small populations (which are drug susceptible)? Two principal differences exist both of which depend on the fact that the passage of time is required for a microbial population to become large Thus the large populations are composed predominantly *of older bacterial cells* and what is perhaps of equal importance the cells have had a greater opportunity to alter their tissue or test tube environment It can be shown both in vitro and in the mou e muscle experimental system that when the age of the population and the environmental alterations pro

duced by growth are divorced experimentally, the large populations of streptococci are as susceptible to penicillin as are the small comparably youthful populations Moreover, when the large "young' populations are placed in *a preformed inflammatory environment in vivo* they retain the penicillin susceptibility characteristic of their youth This observation viewed alone might be taken to mean that age is the determining factor and that the microbial state with respect to penicillin was essentially independent of the *microbial environment* However, it can be shown that the relative drug indifference of the large 'naturally grown microbial populations can be reversed by changing the environment In other words, although it is manifestly impossible to change the age of an individual microbial cell by manipulations of the environment it is quite another matter to change the predominant age of a whole population of cells by appropriate environmental change

A population of microbes growing either in the tissues or in the test tube becomes subject to some inhibitory influence before it reaches the maximal census attainable in those circumstances When the microbial populations become subject to this inhibitory stabilizing influence the microbes also become 'drug indifferent When this environment which has become inhibitory is altered as can be done in vitro by shaking the culture not only do the populations show themselves capable of further growth but also they once again become drug susceptible (Rogers D, 1957) It appears therefore, that it is not the age of *the population per se but its adaptive changes* to the environment it has altered while attaining that age that is associated with its drug indifference In terms of the population as a whole age is a factor but it is a factor which remains responsive to environmental change Indeed the evidence suggests that microbes may be susceptible to penicillin and perhaps certain other drugs only when the microbe is quite young e g less than 4 hours old It should be noted that this is not quite the same concept as the familiar statement that 'penicillin acts only on multiplying bacteria

With the familiar explanation it is implicit that microbial cells in the immediate predivisional state are ipso facto in a state in which they are fully susceptible to penicillin According to the concept proposed herein multiplication or cell division would be only a side aspect of the issue Instead it is postulated that as young microbes grow they lose something or gain something which makes them susceptible to drugs An adaptive mechanism becomes activated, and the capacity thus developed might well remain irreversible in an individual cell When the environment is altered suitably so that a greater number of these older 'adapted cells give birth to younger 'unadapted cells *to the extent that this occurs the population* becomes drug susceptible * In keeping with this concept are recent studies (Park and Strominger 1957 Lederberg 1957) that suggest that penicillin action has to do with cell wall synthesis a happening essentially limited *to newly born microbial cells*

It appears, therefore that it is the microbial state rather than the environment per se that determines drug susceptibility, but the assumption of a particular state by a portion of the microbial population appears to be *specially favored by certain environments* For example, the same diminution of penicillin effectiveness on staphylococci located within leukocytes occurs when the staphylococci are similarly exposed to penicillin in the presence of disrupted leukocytes (Tompsett 1956) When pneumococci are introduced into purulent material they do not show a rapid increase in numbers but persist at the initial census or gradually fall in numbers with the passage of time (W B Wood 1956) It has also been shown that the caseonecrotic lesions of tuberculosis exert a stabilizing and eventual inhibiting effect on the populations of tubercle bacilli therein

One element that is frequently present in the inflammatory necrotic environment of the infection is the presence of a second antimicrobial drug It can easily be shown in vitro (Hobby et al 1942 Jawetz and Gunnison 1953) that the prior or concurrent presence of certain antimicrobial drugs can diminish the susceptibility of some microbial species to the introduction of certain other antimicrobial drugs Moreover in animals the effectiveness of isoniazid *during the early days of* an experimental infection can be largely neutralized by the concurrent administration of nicotinamide or its derivative pyrazinamide (McCune et al, 1956) This phenomenon of the antagonizing influence of one drug on the *effectiveness of another* drug introduced to the environment of the microbe appears to be

* Long before the era of antimicrobial therapy several writers had suggested the concept that bacterial cultures exhibit a period of physiologic youth with changes in morphology and behavior resembling what occurs in more highly differentiated multicellular organisms (e g Sherman and Albus 1924) This subject is discussed on page 143 of *The Bacterial Cell* by R J Dubo (Harvard University Pres 1945)

highly specific for drug pairings and microbial species as will be discussed in a subsequent section

It is important to recognize that the individual microbial species or strains may exhibit considerable differences in the extent to which a particular type of environment may influence susceptibility to a particular drug. Examples of such microbial specificity with respect to drug susceptibility in particular environments were described previously in the discussion of organ difference and the results of environmental manipulations on the drug susceptibilities of the tubercle bacilli in vitro. Another example of the specificity of these phenomena is provided by a comparison of two sets of penicillin studies in mice. Eagle (1952) has shown that in a streptococcal infection of the mouse gastrocnemius muscle a *withholding* of penicillin therapy for 18 or 24 hours after the start of infection results in a marked reduction in the population reducing influence of the penicillin. In experiments with staphylococci (McCune et al 1958) it has been shown that a penicillin susceptible strain infecting the kidney is equally responsive to the population reducing influence of penicillin when therapy was withheld for 54 hours as when the infection and the penicillin therapy *were started together*. In both experiments performed with the same drug inflammatory reaction was present in the tissue when the delayed treatment was started indeed with the staphylococci macroscopic necrotic lesions were present. Moreover in both experiments the population had had full opportunity to age before the institution of therapy. Allowing the infection to proceed for a number of hours before therapy in one tissue infected with one microbial species resulted in a reduction in drug effectiveness. When a similar procedure was followed with the same drug and the same animal but with a different microbial species in a different tissue there was no obvious reduction in drug effectiveness. As the kidney represents the mouse tissue of maximal proliferation of staphylococci (10^9) whereas the populations of streptococci in the mouse muscle are not usually so high (10^6) it is tempting to generalize that the difference in effectiveness of penicillin in the two situations reflected the operation of inhibiting influences on the streptococci in the muscle. However the fact that these questions are considerably more complex may be seen from the fact that in the spleens and the lungs of the animals infected with staphylococci the populations show a fall due to natural influences yet penicillin action was apparently unimpaired

To recapitulate this discussion of microbial states and the role of the environment in their maintenance it can be said that there is convincing evidence that microbial persistence can be caused by the ability of individual microbes to assume a state in which they are relatively insusceptible to an antimicrobial drug. The assumption of this state termed drug indifference is induced or favored by certain environmental influences acting on the microbe. Among these influences are antimicrobial drugs and intermicrobial relationships as well as the influences of the host cellular and humoral defense reactions and the reactions of inflammation. Thus it is believed that it is the adaptive plasticity of the microbe which is the important factor in the influence of the environment on drug effectiveness and not a physical or chemical antagonism exerted by the environment directly on the drug. Instances of the enhancement of drug effectiveness because of environmental adaptation of the microbe also occur. Thus far however such instances have not been associated with the total elimination of the microbes from the host. Therefore to the extent that the subject has been studied the net over all effect on drug effectiveness of the various environmental influences is in the direction of providing situations which favor microbial persistence thus ensuring the survival of a cadre for potential relapse

Genotypic Drug Resistant Microbes

Relapse in the strict sense of the word namely the recurrence of illness in a drug treated infection at some time after the cessation of therapy is virtually always produced by microbes that have survived through the above described phenomenon of microbial persistence. By contrast the relapse during therapy of an apparently controlled infection is almost invariably produced by genotypically drug resistant microbes that have emerged to predominance. In terms of frequency of happening the relapse during therapy produced by the emergence of drug resistant genotypes is quite rare to all intents and purposes its occurrence is limited to three chronic diseases —tuberculosis bacterial endocarditis and certain infections of the kidney and the urinary

tract The *mechanism of the occurrence* of these drug resistant microbial variants has been discussed in the preceding chapter

The individual antimicrobial drugs differ greatly with respect to their association with this genotypically drug resistant form of relapse It is extremely rare with penicillin for example being limited for the most part to cases of enterococcal endocarditis in which even the pretreatment penicillin susceptibility of the microbe is not great In contrast with streptomycin the emergence to predominance of drug resistant forms occurs almost invariably if therapy is prolonged for a sufficient period in the presence of unhealed lesions A particularly dramatic example of this type of relapse was observed in the early experience with streptomycin and miliary tuberculosis (McDermott et al 1947) In 5 patients treated with large daily doses of streptomycin alone a complete disappearance of all evidences of miliary tuberculosis was followed after a short interval by a return of all of the disease manifestations despite the fact that therapy had been uninterrupted Similar observations have been made in cases of staphylococcal endocarditis treated with erythromycin and with other drugs In both the tuberculous and the staphylococcal infections mentioned the microbes associated with the recurring bout of illness were highly resistant in vitro to the drug in use With respect to penicillin and staphylococcal infections it is *not always appreciated that the transformation* of a penicillin susceptible to a penicillin resistant staphylococcal infection during penicillin therapy seldom if ever occurs In other words staphylococcal infections notably those developing in patients in hospitals may be penicillin resistant before any penicillin is administered but if the infection is penicillin susceptible at the start it will remain so

Thus relapse *during therapy* of an infection that was drug susceptible at the start of therapy is least likely to occur with penicillin or chloramphenicol and most likely to occur with streptomycin The tetracyclines erythromycin and novobiocin occupy an intermediate position on this scale Isoniazid appears to occupy a position similar to that of penicillin or chloramphenicol, although there is not universal agreement on this point As the principal offender streptomycin is seldom administered alone the occurrence of relapse during apparently successful therapy as a consequence of the emergence to predominance of drug resistant genotypes represents a relatively rare phenomenon in clinical practice

Treatment Failures as Distinguished from Relapse

Although it is rare to observe the reappearance of illness during therapy after its disappearance in the early days of therapy, it is less rare to encounter treatment 'failures' or "incomplete successes" associated with the appearance of drug resistant microbes This phenomenon may occur in any chronic destructive *infection*, but to all intents and purposes it is limited to chronic pulmonary tuberculosis

The cases usually appear in one of two forms with gradations between In one form there is extensive caseonecrotic involvement of a large area of lung The patient may show some slight systemic improvement during the early weeks of therapy However no significant roentgenographic changes occur tubercle bacilli are constantly discharged in the sputum and eventually the bacilli are predominantly resistant in vitro to the antimicrobial drugs that have been administered In the other form an area of apparently exudative disease surrounds a cavity The pulmonary infiltration regresses satisfactorily during the early stages of therapy but the cavity persists, the discharge of tubercle bacilli continues and eventually these bacilli likewise are predominantly drug resistant in vitro

Many of these cases especially the second variety, do not actually represent treatment failures in the sense that the disease has never been influenced or is resuming an actively progressive character after only a temporary inhibition by the chemotherapy Instead particularly if isoniazid is included in the therapy the disease appears to be held localized yet it obviously remains unhealed Empirically it is known that a tuberculous cavity that persists during prolonged antimicrobial therapy presents the precise set of circumstances that favor the emergence to predominance of drug resistant tubercle bacilli However the mechanisms responsible for this situation are not clear

It is known that the caseous tuberculous lesion cut off from a bronchial communication represents an environment relatively unfavorable for the proliferation of tubercle bacilli However this situation is reversed when a cavity is formed through bronchial communication the caseous lining of the cavity then provides a most favorable environment for the bacilli By this process of opening a bronchial

communication the environment of the tubercle bacilli is changed abruptly from one generally favorable for microbial states of drug indifference to one generally more favorable for maximal microbial growth The latter circumstances should be especially propitious for the action of antimicrobial drugs Hence on the one hand there should be a marked drug induced incapacitation of the microbial population resulting in a tendency toward healing while on the other hand conditions should be ideal for the selection of drug resistant microbes In actuality this is indeed what happens an appreciable percentage of tuberculous cavities (10 to 20%) are now showing complete healing without closure on prolonged chemotherapy and in the cases in which the discharge of tubercle bacilli in the sputum persists the bacilli are predominantly drug resistant

It is conceivable therefore that with long continued chemotherapy the failure of a cavity to *close* as opposed to its failure to *heal* may be more a cause than a consequence of the emergence of *drug resistant tubercle* bacilli The implications of the continued discharge of the drug resistant tubercle bacilli and the use of two or more drugs to postpone or prevent their emergence to predominance will be discussed in a subsequent section

Relation of Drug Resistance to Microbial Persistence

From the foregoing it is clear that there are at least two ways whereby microbes can survive the impact of antimicrobial drugs within the body by environmental adaptations favoring drug indifference and by the production of drug resistant genotypes Of the two mechanisms the adaptive one is by far of the greater *importance numerically in that it provides the* principal basis for the post treatment relapse of acute and chronic infections it permits the survival of microbes until subsequent environmental change provides conditions favorable for the production of drug resistant genotypes and it is largely responsible for balking attempts at chemoprophylaxis Both the adaptive and the genotypic phenomena are easily distinguishable from each other and in general they are observable as separate processes However some notion of the complexity of drug microbe relations in vivo is provided by the fact that there is convincing evidence that a microbe can be genetically drug resistant and yet persist in the tissues in a state resembling drug indifference

In general as described above populations of drug susceptible microbes decline to a low census in the tissue during drug therapy and then persist there in a drug susceptible state If drug resistant variants emerge they increase in numbers despite the chemotherapy and the population curves are essentially the same as in infected animals that receive no treatment However in experiments in mice with both tubercle bacilli and staphylococci it has been noted that this up-surge from a small surviving drug resistant population does not invariably occur Instead in a few instances the microbes continue to persist at a low census throughout the period of drug therapy in a fashion identical with that of the drug susceptible persisters

These observations suggest there are aspects and implications of the adaptive states of drug indifference which have nothing to do with the drugs To state this is merely to say that microbial populations (including the drug resistant cells therein) can become dormant or truly latent in an animal host by the *operation of natural forces* and not only as a result of antimicrobial therapy In short the mechanisms of microbial persistence during drug therapy are probably very much like the mechanisms which permit infections to become dormant or latent in the absence of therapy

It is beyond the scope of the present discussion to consider the host mechanisms possibly operative in maintaining an infection in the dormant or latent state It is sufficient to emphasize that probably there has been too much of a tendency to regard the parasite that is actively producing disease as being very much the same physiologically and morphologically as when it is present in a dormant or latent infection Viewed in these terms the evocation of a latent infection requires nothing more than a momentary lapse in bodily defenses whereas in actuality it probably also requires very considerable environmental adaptive changes on the part of the parasite

Physiologic Imprisonment of Dormant or Latent Infections

An important question is whether the continued administration of an *antimicrobial drug* exerts any influence at all in maintaining an

infection in the dormant or latent state In short can microbes that are not sufficiently active metabolically to produce disease be drug susceptible? Certain clinical observations suggest that the host parasite reaction must evolve to a certain degree of maturity before antimicrobial drugs become fully effective The evolution of an infection to this point can be prevented for long periods, perhaps indefinitely, by the administration of appropriate drug therapy In these circumstances, therefore the microbial population is in the paradoxical situation of being both drug influenced and drug indifferent' at the same time It is probable in this situation, that a portion of the microbial population is and remains drug indifferent If members of this drug indifferent population assume a state of greater drug susceptibility or are newborn into such a state they should be incapacitated immediately by the antimicrobial drug present in the environment Therefore the infecting population considered as a whole may be said to be in a state of *physiologic imprisonment* The individual microbes are not eradicated neither are they permitted to evolve to a situation in which drug activity could be more definitive

Physiologic imprisonment thus presumably characterizes the status of infections both when a beginning infection is held abortive under the influence of chemoprophylaxis and when a fully developed disease has entered into a clinical remission as a result of the initiation of antimicrobial therapy In both situations (except for the rare 'escape due to drug resistant genotypes) the infection remains dormant or latent for so long as the antimicrobial therapy is continued In the period thereafter the persisting microbes may continue to remain dormant or latent for the entire lifetime of the host for some shorter period culminating in microbial death due to natural forces or until some appropriate combination of changes in both bodily defenses and microbial adaptations leads to evocation of the infection to the point of clinical relapse

In most of the preceding discussion the principal emphasis has been on the possible mechanisms whereby microbes can survive drug exposure in vivo and hence be available for post treatment relapse This extensive consideration of the failures of antimicrobial therapy rather than its successes is obviously disproportionate to the relative frequency of the two phenomena It is believed however, that the possible mechanisms of the failures lend themselves to more extended scrutiny and thus provide better understanding of the mechanisms of therapeutic success Evocation to relapse is only one of the possible fates of post treatment microbial persisters as outlined in the preceding paragraph An important question at issue, therefore is whether the occurrence of certain of the other types of ultimate outcome could be favored by any practicable manipulations of time dose relationships Would continued treatment with the resulting prolonged physiologic imprisonment' have effects not present following shorter periods of therapy? About all that can be said is that in general, the longer the period of postillness administration of chemotherapy, the less likely are the chances of relapse

Time Dose Relationships

Consideration of the proper time dose relationships must be made against the background of both the drug microbe and the drug host interreactions as they presumably occur in vivo

A prominent question in clinical practice in the case of any one drug is the size of the concentration necessary in the body fluids and the length of the period it should be present to ensure maximally effective therapeutic action It is probable as mentioned previously that the size of this effective concentration is no greater than would be necessary under reasonably similar conditions for the inhibition of the same microbe in vitro As neither the quantity of drug which survives passage through the host to the site of the lesion nor the physiologic state of the microbe is readily determinable the effective concentration in actuality represents an abstraction

In abstract terms therefore the maximally effective concentration of an antimicrobial drug represents that concentration increments above which serve no useful purpose per unit of time

The problem thus consists of determining what portion of the 24 hour period (or any other arbitrary unit of time) must be occupied by an 'effective concentration' of a particular drug for the ideal therapy of a particular infection Another aspect of the same problem

is what should be the length of the intervals between periods of effective concentration The respective lengths of these time fractions presumably differ considerably among various drugs and various diseases depending upon a number of factors Prominent among these are the relative importance to the economy of the microbe of the particular metabolic processes interfered with by the drug i e its degree of *activity* the effects of less than effective concentrations which by the very *nature of the ca e are present for measurable* periods after the period of effective concentration the speed with which resurgence of microbial growth occurs following exposure to an effective concentration i e the extent to which drug action is reversible by the parasite and finally the speed with which effective host defenses can become operative

The most important of these factors would seem to be the activity or degree of effectiveness of the drug i e the extent to which the infectin population can be incapacitated or reduced in censu durin any one period when an effective concentration is present As discussed in the preceding section the activity of a drug against a particular infection is a *complicated question and one in which* oversimplifications are alluring Nevertheless examination of the results obtainable from use of the same drug in different infections provides information of relevance in regard to this question

For example the concentrations of penicillin necessary for inhibition of pneumococcus in vitro are sub tantially lower than tho e necessary for inhibition of *M aureus* or the non hemolytic streptococci that produce endocarditis With pneumococcal pneumonia a period of effective concentration of penicillin of 4 to 6 hours of each 24 hour period is adequate for satisfactory therapy In staphylococcal infections with bacteremia or in nonhemolytic streptococcal endocarditis the corresponding period of effective concentration of the same drug is 20 hours or more

The peed with which resurgence of microbial growth occurs *following the presence of* an effective concentration for certain periods obviously bears some relationship to the proper length of both the periods and the intervals between them It can be shown under appropriate conditions in vitro that bacteria which have survived 3 or 4 hours of drug exposure do not immediately *resume growth* when the drug in the environment has been neutralized A *similar* phenomenon presumably occurs in vivo but whether it occurs on a significantly *wide scale at any one time is not known* A related factor is the usual generation time of a microbial species but it is probable that this particular factor has been emphasized disproportionately For example in the illustrations cited above there is no reason to believe that the streptococcal or staphylococcal infections are any more fulminatin in the sense that multiplication of the parasites occurs at a more rapid rate following a period of drug exposure than is the case with pneumococcus In a very *general way therefore the generation time of* the parasite is a factor which influences the length of the proper periods of effective concentration of drug and the intervals between them A more influential factor however is the degree of incapacitation produced in the members of a particular microbial pecies by a particular drug

The extent to which the host can tolerate resumed microbial activity without exhibiting clinical illness is another factor which has an obvious influence on the proper length of the interval between periods of effective concentrations of drug Thus with tuberculosis and syphilis maintenance of an effective concentration of drug for a period of only 5 or 6 hours every 2 or 3 days apparently results in satisfactory treatment It should be emphasized however that such treatment regimens do not necessarily represent the maximally effective therapy and appreciable but clinically undetectable tissue destruction might be occurring during their use

Thus it appears that the absolutely continuous presence of an *effective* concentration of any one of the available drugs is unnecessary for satisfactory antimicrobial therapy With a relatively insusceptible microbial species and particularly *one whose increased metabolic* activity is almost immediately reflected by the appearance of clinical illness the intervals between *periods of effective* concentration of drug must be considerably shorter than with *highly susceptible microbes or in chronic* infections

Multiple Drug Therapy and Drug Resistance

Consideration of time dose relationships would be incomplete without recognition of the fact that in clinical practice two or mo e drugs are frequently administered together This multiple dru therapy is usually given in an attempt to accomplish *one of two purposes*

(1) to bring the infection under control more effectively than would be the case with the use of one drug alone (2) to prolong the total period during which some antimicrobial influence is being supplied There is an obvious overlapping in these purposes, but what might accomplish one purpose might not necessarily accomplish the other

At the outset it should be noted that despite the many reports indicating the value of various antimicrobial drug pairings based on observations in vitro the examples of apparently beneficial effects from multiple drug therapy in man are remarkably few Indeed, to all intents and purposes the examples are limited to infections with enterococci or with tubercle bacilli The inclusion of brucella infections in this category is debatable Conversely, there is at least one protozoan infection, malaria in which apparent benefits from multiple drug therapy have been observed

The use of streptomycin and penicillin in enterococcal infection notably endocarditis represents the one acceptable example of enhancement of the total antimicrobial influence per unit of time by the concurrent administration of two drugs With either drug administered alone in the highest tolerable doses only partial remissions are obtainable whereas when both drugs are given together the remissions are usually complete (Robbins and Tompsett 1951) This effect could be caused by action of the two dissimilar chemicals at different sites in the same microbial cell by the action of one drug on microbes in a state indifferent to the other drug or by the action of one drug on microbes genotypically resistant to the other drug From observations in humans and studies in vitro it is not clear whether all these mechanisms are operative or whether only one mechanism is the principal one As the microbe is relatively insusceptible to each drug when tested in vitro and quite susceptible to the pair together it is probable that both drugs do enter into reactions with the same microbial cell this obviously does not exclude the possible concurrent operation of the other two possible mechanisms

With the antimicrobial drugs available today enterococcal infections represent one of the few situations in which a complete remission of the illness is not produced by the administration of only one drug In most other infections therefore even if two drugs together were to provide an increase in total antimicrobial influence, the fact would be virtually impossible to demonstrate clinically because the same clinical result would be attainable by the use of one drug alone Thus the area of advantage attainable by enhancement of total antimicrobial effect per unit of time would be principally limited to effects on microbial persistence and post treatment relapse It is well established that microbial persistence per se is not significantly influenced by multiple drug therapy The question of the relative incidence of post treatment relapse has not been studied in detail However from experience with endocarditis caused by viridans streptococci and with brucellosis it can be stated that relapse occurs following multiple drug therapy and apparently occurs in approximately the same incidence as when the more powerful of the two drugs employed is administered alone The only indication of a possible difference has been that with the multiple drug regimens employed (streptomycin penicillin for endocarditis streptomycin tetracycline for brucellosis) the relapse rate is essentially the same after 2 weeks of therapy as when penicillin or tetracycline is used alone for longer periods As the actual incidence of relapse does not seem to be affected the disadvantages attendant on the use of streptomycin in these regimens make this possible enhancement per unit of time of doubtful practical importance

On the basis of the demonstrated superiority of the streptomycin penicillin multiple drug regimen in enterococcal infections and studies with the enumeration technic in mice there is some reason to believe that streptomycin along with penicillin may provide enhanced total antimicrobial activity in penicillin susceptible staphylococcal infections in man It is important to realize however that a superiority of this 2 drug regimen over penicillin alone in this situation has never been actually demonstrated in man and indeed it would be difficult to do so The other indication for multiple drug therapy—attempts to interfere with the emergence of drug resistant microbes—is not applicable in penicillin susceptible staphylococcal infections for reasons discussed in a preceding section With other antistaphylococcal drugs e g erythromycin this second indication for multiple drug therapy would be applicable

Thus except for enterococcal infections (and perhaps also for staphylococcal infections) there is no convincing evidence from clinical observation that the use of two drugs together increases the total antimicrobial effect

per unit of time However there is one bit of clinical evidence that suggests that the beneficial effects of using PAS as a companion drug to streptomycin are accomplished by enhancement of the total antimicrobial effect per unit of time For the use of PAS along with streptomycin did seem to be associated with a slightly lowered incidence of the emergence of meningitis in drug treated miliary tuberculosis although this complication was by no means abolished In experimentally infected mice it can be shown that para aminosalicylic acid increases the total antimicrobial influence per unit of time when used with streptomycin Para aminosalicylic acid does not similarly enhance the activity of isoniazid when chemotherapy is started at the time of infection However with the different microbial environmental situation provided by a tuberculous infection that has been allowed to progress untreated for 21 days the addition of PAS to isoniazid does enhance the total antimicrobial influence exerted by the chemotherapy In the same experimental model it can be shown that several substances notably isoniazid greatly enhance the total antimicrobial influence when administered with the nicotinamide derivative pyrazinamide For reasons discussed elsewhere it has not yet been possible to demonstrate enhancement of total antimicrobial influence per unit of time in tuberculous infections in man by adding other drugs to isoniazid regimens (McDermott et al 1954)

The paucity of these examples of the superiority of multiple drug therapy from the standpoint of *enhancement* and the prominence of streptomycin in the few examples which exist suggests that superior antimicrobial effectiveness produced by multiple drug administration *is a highly speciali ed phenomenon* By this is meant that enhancement undoubtedly occurs but it occurs only with certain drug pairings on certain microbial species The phenomenon is apparently not to be expected with the same drug pairing and a microbe of another species even though the latter may be susceptible to both drugs in the pairing It is also of considerable interest that the most definite examples of 2 drug summative effects that have been observed in laboratory animals or in man have involved either streptomycin or pyrazinamide Each of these drugs exerts widely different effects on certain microbes depending on the conditions of the environment This suggests that much less effort should be devoted to testing multiple drug combinations in standard media under phy i ologic conditions and more effort devoted to defining the drug susceptibilities of the individual microbial species under conditions that simulate their environment in vivo

The case that multiple drug therapy can lead to a *prolongation* of the period during which some antimicrobial influence is being exerted is based almost entirely on observations in the treatment of tuberculo is To a certain extent this happened because the first major antituberculous drug was streptomycin On the one hand streptomycin is apparently one of the most effective companion drugs in multiple drug therapy on the other hand it ranks first among the major drugs with respect to eventual loss of effectiveness because of the emergence to predominance of drug resistant microbes The fact that both of these properties so germane to consideration of multiple drug therapy are displayed in such an accentuated fashion by streptomycin had the healthy effect of focusing attention at an early stage on the questions of drug resistance and multiple drug therapy in tuberculosis However this same situation also led to certain generalizations on the chemotherapy of tuberculosis with isoniazid which with longer experience appear to have been unwarranted

Obviously the question of the possibility of prolonging the period during which an antimicrobial drug can maintain ome effectiveness arises only when the effectiveness of that drug tends to become neutralized with the passage of time With the antimicrobial drugs available at present drug neutralization occurs only as a result of the emergence of otherwise typical genotypic drug resistant microbes in other forms of chemotherapy for example insulin therapy the eventual impairment of drug effectiveness can be accomplished by inactivation mechanisms mobilized by the host The fact that the antimicrobial effectiveness of streptomycin on tubercle bacilli is frequently neutralized by the emergence of drug resistant bacilli was clearly established by the early studies of miliary tuberculosis Thus the streptomycin tubercle bacilli relationship provides a wholly satisfying model for consideration of the possible prolongation of drug effectiveness either by alterations in time do e relationships or by the addition of companion drugs

One way to retard the emergence to predominance of drug resistant microbes is to administer the drug in such a way that considerably less than the maximally effective concentration is present per unit of time This was done with streptomycin The early regimens consisted of a total dose of approximately 60 mg per Kg of body weight administered

(1) to bring the infection under control more effectively than would be the case with the use of one drug alone, (2) to prolong the total period during which some antimicrobial influence is being supplied There is an obvious overlapping in these purposes but what might accomplish one purpose might not necessarily accomplish the other

At the outset it should be noted that despite the many reports indicating the value of various antimicrobial drug pairings based on observations in vitro the examples of apparently beneficial effects from multiple drug therapy in man are remarkably few Indeed to all intents and purposes the examples are limited to infections with enterococci or with tubercle bacilli The inclusion of brucella infections in this category is debatable Conversely there is at least one protozoan infection malaria in which apparent benefits from multiple drug therapy have been observed

The use of streptomycin and penicillin in enterococcal infections notably endocarditis represents the one acceptable example of enhancement of the total antimicrobial influence per unit of time by the concurrent administration of two drugs With either drug administered alone in the highest tolerable doses only partial remissions are obtainable whereas when both drugs are given together the remissions are usually complete (Robbins and Tompsett 1951) This effect could be caused by action of the two dissimilar chemicals at different sites in the same microbial cell by the action of one drug on microbes in a state 'indifferent to the other drug or by the action of one drug on microbes genotypically resistant to the other drug From observations in humans and studies in vitro it is not clear whether all these mechanisms are operative or whether only one mechanism is the principal one As the microbe is relatively insusceptible to each drug when tested in vitro and quite susceptible to the pair together it is probable that both drugs do enter into reactions with the same microbial cell this obviously does not exclude the possible concurrent operation of the other two possible mechanisms

With the antimicrobial drugs available today enterococcal infections represent one of the few situations in which a complete remission of the illness is not produced by the administration of only one drug In most other infections th refore even if two drugs together were to provide an increase in total antimicrobial influence, the fact would be virtually impossible to demonstrate clinically because the same clinical result would be attainable by the use of one drug alone Thus the area of advantage attainable by enhancement of total antimicrobial effect per unit of time would be principally limited to effects on microbial persistence and post treatment relapse It is well established that microbial persistence per se is not significantly influenced by multiple drug therapy The question of the relative incidence of post treatment relapse has not been studied in detail However, from experience with endocarditis caused by viridans streptococci and with brucellosis, it can be stated that relapse occurs following multiple drug therapy and apparently occurs in approximately the same incidence as when the more powerful of the two drugs employed is administered alone The only indication of a possible difference has been that with the multiple drug regimens employed (streptomycin penicillin for endocarditis streptomycin tetracycline for brucellosis) the relapse rate is essentially the same after 2 weeks of therapy as when penicillin or tetracycline is used alone for longer periods As the actual incidence of relapse does not seem to be affected the disadvantages attendant on the use of streptomycin in these regimens make this possible enhancement per unit of time of doubtful practical importance

On the basis of the demonstrated superiority of the streptomycin penicillin multiple drug regimen in enterococcal infections and studies with the enumeration technic in mice there is some reason to believe that streptomycin along with penicillin may provide enhanced total antimicrobial activity in penicillin susceptible staphylococcal infections in man It is important to realize however that a superiority of this 2 drug regimen over penicillin alone in this situation has never been actually demonstrated in man and indeed it would be difficult to do so The other indication for multiple drug therapy—attempts to interfere with the emergence of drug resistant microbes—is not applicable in penicillin susceptible staphylococcal infections for reasons discussed in a preceding section With other antistaphylococcal drugs e g erythromycin this second indication for multiple drug therapy would be applicable

Thus except for enterococcal infections (and perhaps also for staphylococcal infections) there is no convincing evidence from clinical observation that the use of two drugs together increases the total antimicrobial effect

dence that it exerts any unusual activity on genotypic streptomycin resistant cells More over when used alone in experimental tuber culous infections in mice the relatively slight but definite degree of antimicrobial action of the PAS is short lived Within 3 weeks of the start of therapy the populations of tubercle bacilli appear to escape from the inhibitory influence of the PAS and despite the continu ation of PAS administration the populations of tubercle bacilli increa e to the same census as in the untreated control animals Conse quently if as appears likely there is some thing specific about the PAS tubercle bacillus streptomycin relationship it suggests that the same microbial cell under the influence of one of these drugs has an altered reactivity to the other drug In short it appears likely that the inhibition of an individual tubercle bacillus exposed to streptomycin PAS proceeds through a different set of biochemical reactions than an individual tubercle bacillus inhibited by streptomycin alone The fact that tubercle bacilli under the influence of one drug can react to another drug in a way different from their reaction to the second drug used singly has been clearly demonstrated both in vivo and in vitro (McCune et al 1956 Koch Weser et al 1956)

If the above concept of simultaneous action of PAS and streptomycin on the same tubercle bacillus has validity it follows that the demon strated superiority of streptomycin PAS to streptomycin alone in experimental tuber culous infections (and the apparent superiority in man) depends upon *the streptomycin sus ceptible cells in the population being acted upon differently and more effectively per unit of time* The genotypic streptomycin resistant cells in the population would be exposed only to that relatively weak and poorly sustained action provided by PAS alone Therefore it would be expected that if the lesions with a patent bronchial communication remain un healed the streptomycin resistant genotypes would eventually emerge to predominance and in actuality this is exactly what happens Indeed it has been observed by Amberson et al that in a small series of patients treated with streptomycin PAS in conditions most fa vorable to the emergence of streptomycin re sistant tubercle bacilli (i e acute caseating pneumonia of lobar extent with the strepto mycin administration daily) the emergence to predominance of such strains occurred within a 3 or 4 month period in approximately one third of the patients Fortunately this type of disease represents a relatively mall minority of the ca es of pulmonary tuberculosis hence the over all results with streptomycin PAS were appreciably better than those obtained in acute caseating pneumonia during the period before other antituberculous drugs were avail able The extensive reductions in streptomycin dosage at the time of introduction of PAS as a companion drug also presumably contributed to the better results attained from this mul tiple-drug therapy If the concept that the principal effect of PAS and streptomycin is on the same streptomycin susceptible tubercle bacilli has validity it would also be expected that some of the microbial persisters following completion of streptomycin PAS therapy would be genotypically drug resistant in vitro In actuality this is the case as may be seen from the presence of drug resistant tubercle bacilli in some of the solid pulmonary lesions of the closed type that have been resected in patients who have received streptomycin PAS Moreover as discussed in a previous sec tion a relatively small minority of the persist ing tubercle bacilli in drug treated mice are resistant in vitro to the drug employed

To recapitulate the superiority of strepto mycin PAS to streptomycin alone in tuber culous infections does not appear to represent so much an actual *prolongation* of the period during which streptomycin maintains effec tiveness as it does a greater degree of effec tiveness of both drugs during the early months of treatment In effect therefore in the pres ence of streptomycin susceptible tubercle bacilli exposed to PAS the streptomycin be haves more as a drug such as penicillin to which the emergence of drug resistant cells during therapy is less of a problem

For the reasons set forth above and not ably the relatively weak and short lived in fluence of PAS used singly in experimental tuberculous infections it seems unlikely that the chief role of PAS in streptomycin PAS therapy is to exert a long-continued low level action on the streptomycin survivors thereby maintaining the gains made originally and principally by the streptomycin It must be recognized however that in tuberculous in fections in humans it would be very difficult and probably impossible to distingui h this type of continued drug influence from an in fluence produced by an actual prolongation of the period of effectiveness of the primary drug The various antimicrobial drugs differ mark edly in the extent to which drug resistant genotypes of a single microbial pecies will emerge Consequently the concurrent admin istration of a weak drug with long term effec

in 6 equal portions at equal intervals throughout each 24 hour period It was these regimens that were associated with such a high incidence of drug neutralization due to drug resistant microbes Because of this and in an effort to reduce drug toxicity the dosage was steadily lowered The standard regimen employed was 20 mg per Kg administered in only a single dose each day A further reduction occurred especially in the United States when daily administration was largely abandoned and the 20 mg per Kg dosage was administered only 2 or 3 times each week As these successive reductions in dosage coincided with the introduction of para aminosalicylic acid as a companion drug evaluation of the precise role played by the PAS *at the level of prolonging the period of effectiveness of the streptomycin* has been clouded This is not to say that the effectiveness of PAS as a companion drug for streptomycin has not been established What is not completely established however is whether the use of the PAS actually prolongs the period during which the streptomycin remains effective

The addition of a companion drug such as PAS could affect the susceptibility of a microbial population to a primary drug such as streptomycin through several possible mechanisms

The companion drug could act principally on the relatively few microbes resistant to the primary drug that would be present at the time multiple drug therapy was started (or be born thereafter) and bring about their death or favor their assumption of a state of microbial persistence

The companion drug could act principally on microbes situated in environments that specially favored states of drug indifference to the primary drug

The companion drug could act on the same microbial cells that were also being influenced by the primary drug producing a total inhibitory influence on the individual cell of a type different from that exerted by the primary drug alone

Finally the companion drug could *appear* to be prolonging susceptibility to the primary drug when in actuality it was serving merely to maintain gains made earlier by the primary drug

Unfortunately the available information is too scanty to permit more than free speculations as to the probable occurrences in the infected host treated with two drugs together such as PAS and streptomycin A priori there is no reason to doubt that more than one of the postulated mechanisms might not be operating at the same time In the specific case of PAS and streptomycin there is ome reason to be skeptical that the principal role of the PAS *is to exert action on the cells* resistant or indifferent to the streptomycin The concept of an action of one drug on cells indifferent to the other is alluring in view of the evidence cited previously that tubercle bacilli *may display* a considerable difference in susceptibility to a particular drug depending upon environmental circumstances It is known that PAS is ineffective against populations of tubercle bacilli in vitro unless the initial microbial census is relatively low This fact and possibly also the fact that PAS is virtually ineffective against tubercle bacilli situated within monocytes in vitro do not indicate any unusual action of the drug on cells metabolizing at less than maximal capacity Nevertheless the possibility can not be excluded that tubercle bacilli in an environment such as that of a caseous lesion might be more susceptible to PAS than to streptomycin Much more information is needed on this question of PAS tubercle bacilli relationships in different environments

The concept that the principal PAS action is on streptomycin resistant genotypes is probably the one most frequently presented and is *based in its essentials on the assumption of an* independent action of the two drugs on different microbial cells If this were true it should not matter *which* drugs or which microbial *species were involved so long as both drugs* were capable of exerting an inhibitory action (in the presence of each other) on the cells of the particular microbial species Although precise studies are not available there is some reason to doubt from clinical experience that a companion drug effective on *E coli* for example significantly postpones the emergence to predominance of streptomycin resistant *E coli* in infections of the urinary tract Likewise in tuberculous infections treated with either of the drug pairings isoniazid streptomycin or isoniazid PAS the PAS given *daily* appears *to be more effective as a companion drug to* the isoniazid than the intrinsically more powerful therapy of streptomycin administered twice weekly These bits of evidence are admittedly flimsy Nevertheless they point in the same direction as the more solid evidence on drug pairings and enhancement of total antimicrobial activity presented above namely that the few acceptable examples appear to represent highly specific phenomena The drug PAS is a relatively weak inhibitor of *M tuberculosis* both in vitro and in vivo and there is no evi

administration of the drug the census of culturable bacilli eventually shows a marked increase from the previous low level and the freely proliferating population is drug resistant in vitro When isoniazid is given along with the pyrazinamide or when it is given immediately prior to the pyrazinamide the infection does not relapse but instead vanishes into a state of true latency Moreover this transformation into a latent infection can occur even though there are some genotypic pyrazinamide resistant cells in the residual latent infection *Thus a drug pairing that fails to eliminate all drug resistant microbial cells from the host nevertheless abolishes the phenomenon of relapse during treatment due to the emergence of drug resistant microbes* An essentially similar result can be produced by giving streptovaricin along with isoniazid in the same experimental model It is of particular interest that streptovaricin like PAS is a relatively weak antituberculous agent when employed alone and also like PAS eventually lose even its slight effectiveness by virtue of the emergence of streptovaricin resistant cells Yet when streptovaricin is given with isoniazid the resultant total antimicrobial influence is surpassed only by pyrazinamide isoniazid and is markedly superior to that attained with isoniazid alone

The situation with respect to the use of two or more antimicrobial drugs together can be summed up thus infections with enterococci or with tubercle bacilli represent the only examples available from present clinical experience in which there is convincing evidence that certain drug pairings produce results superior to those attainable when the more powerful of the two drugs is used alone With certain other infections notably those produced by brucella or staphylococci there is some evidence of superior 2 drug effectiveness in certain circumstances in laboratory animals but the same type of drug effectiveness in humans has not yet been demonstrated When the evidence available both from studies in laboratory animals and in man is reviewed certain inferences seem to be reasonable

(1) The superiority of a drug pairing to one of its members is a phenomenon with considerable specificity in terms of the drugs and microbial species involved hence superior drug pairings are relatively rare and are not to be expected with any pairing and any drug susceptible parasite (2) The superior effectiveness of the pairing appears to depend principally on the action of both drugs on the microbes susceptible to both drugs and hence in large measure consists of both drugs acting on the same microbial cell (3) The influence of multiple drug regimens on the emergence to predominance of drug resistant microbes is indirect and is a consequence of this greater inhibitory action produced on the microbes susceptible to both drugs It is doubtful that an action of one drug on the microbes genotypically resistant to the other drug plays an important role in the process (4) Even with the few microbial species for which multiple drug action has been established as superior no combination of drugs has yet been found which will *uniformly* eliminate all of the drug susceptible microbes from the infected tissues of the host

Influence of Antimicrobial Therapy on Immunity

The participation of the host has been discussed principally from the standpoints of host mechanisms for the neutralization of drug activity on the one hand and the capacity of the host to render the effects of antimicrobial therapy durable on the other hand There is another highly important aspect of host participation namely to what extent can the customary responses of the host to an infection be significantly modified by therapy It must be remembered in this connection that however valuable host influences may be in maintaining final control of an infection the responses to the stimulus of infection are *ad hoc* in nature and are not invariably beneficial to the host as a whole organism

Though there is relatively little certain information on this subject it can be said at the outset that such influence as the currently available drugs may exert on the host response to infection is apparently accomplished indirectly by the action of drug on parasite and not by any direct interference of drug on the mechanisms of immunity Examples of such an indirect action of antimicrobial therapy on host resistance are provided by the results of the chemotherapy of streptococcal pharyngitis syphilis scrub typhus and Q fever

The principal dangers from untreated streptococcal pharyngitis (Group A beta hemo

tiveness and a powerful drug with short lived effectiveness (because of drug resistant genotypes) could produce the appearance and certain of the benefits of an extended period of multiple drug action without this actually being the case

Evaluation of the exact role of isoniazid in the multiple drug therapy of tuberculosis is difficult because unlike the situation with streptomycin there appears to be a marked disparity between the appearance of isoniazid resistant tubercle bacilli on the one hand and their potential for producing relapse in drug treated patients on the other hand Certainly, the neutralization of drug effectiveness due to drug resistance that was demonstrated so dramatically in miliary tuberculosis with streptomycin does not occur with isoniazid Moreover most strains of isoniazid resistant tubercle bacilli show alterations in other biologic characteristics including a rather marked reduction in pathogenicity for guinea pigs and monkeys These phenomena are considered in detail in Chapter 11 of this volume (pp 277 309) Because of the uncertainties concerning the significance of isoniazid resistant tubercle bacilli there has been very little extensive clinical study of the long term use of isoniazid alone Without this information when dealing with so powerful a drug as isoniazid it is difficult to form conclusions concerning the relative degree of effectiveness of any companion drug

In an experimental model that permits measurement of multiple-drug effects in mice streptomycin and isoniazid administered together produce a greater total antimicrobial influence than the more powerful drug (isoniazid) when used alone In the same system the administration of PAS with the isoniazid does not increase the total antimicrobial influence when the chemotherapy is started at the beginning of the infection However when the infection is allowed to progress for 21 days before starting therapy the isoniazid shows a somewhat lessened influence and the addition of PAS produces a slight but definite increase in the total antimicrobial effect From experience in the treatment of tuberculosis in man it is quite generally accepted that both isoniazid streptomycin and isoniazid PAS represent more powerful chemotherapies than isoniazid alone However as mentioned above the lack of satisfactory comparative data makes it impossible to evaluate the margin of the superiority of the 2 drug regimens Whether the increased effectiveness of these 2 drug regimens is related to effects on the emergence of isoniazid resistant tubercle bacilli also cannot be judged because of the uncertainties concerning the relapse potential of isoniazid resistant tubercle bacilli during continued treatment with the drug It is the belief of the present writer that isoniazid usually continues to exert an antimicrobial influence on tubercle bacilli throughout prolonged periods of administration Consequently it is also believed that the superiority of the 2 drug regimens employing isoniazid represents the phenomena of enhanced antimicrobial influence per unit of time rather than an influence tending to make the infecting population remain isoniazid susceptible Considerations of this question in detail have been presented elsewhere (Deuschle et al 1954 McDermott 1957) It should also be mentioned with respect to the specific case of isoniazid PAS that it is believed by some investigators that PAS can hamper the bodily inactivation of isoniazid to some extent This point cannot yet be considered as having been definitely established

As in the case of streptomycin PAS even if the principal influence of drug pairings is exerted on the microbial cells susceptible to both drugs it does not follow that the emergence to predominance of drug resistant genotypes would necessarily remain unaffected On the contrary there should be impressive reductions in the relapse that occurs during treatment due to the emergence of drug resistant microbes For the enhanced drug effect on the doubly susceptible microbial cells produced by the two drugs together should result in a greater degree of incapacitation of the majority of the infecting population The resulting reduction in microbial proliferation would lessen the chances of the birth of drug resistant genotypes Moreover the greater control of the infection should considerably facilitate the processes of healing and hence modify the tissue environment toward one less conducive to the emergence of drug resistant microbes in large numbers

Examples of such an influence on the emergence of drug resistant tubercle bacilli have been observed in experiments in mice treated with pyrazinamide isoniazid or streptomycin isoniazid There is an experimental basis for believing that when pyrazinamide and isoniazid are administered together the principal action of the two drugs is exerted on the same microbial cells When pyrazinamide is employed alone it produces a marked drop in the census of culturable tubercle bacilli in the mouse tissues However despite continued

administration of the drug the census of culturable bacilli eventually shows a marked increase from the previous low level and the freely proliferating population is drug resistant in vitro When isoniazid is given along with the pyrazinamide or when it is given immediately prior to the pyrazinamide the infection does not relapse but instead vanishes into a state of true latency Moreover this transformation into a latent infection can occur even though there are some genotypic pyrazinamide resistant cells in the residual latent infection *Thus a drug pairing that fails to eliminate all drug resistant microbial cells from the host nevertheless abolishes the phenomenon of relapse during treatment due to the emergence of drug resistant microbes* An essentially similar result can be produced by giving streptovaricin along with isoniazid in the same experimental model It is of particular interest that streptovaricin like PAS is a relatively weak antituberculous agent when employed alone and also like PAS eventually lose even its slight effectiveness by virtue of the emergence of streptovaricin resistant cells Yet when streptovaricin is given with isoniazid the resultant total antimicrobial influence is surpassed only by pyrazinamide isoniazid and is markedly superior to that attained with isoniazid alone

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The principal dangers from untreated streptococcal pharyngitis (Group A beta hemo-

lytic) are the possibility of spread of infection by direct extension, and less frequently, the possible development of the nonsuppurative complications such as rheumatic fever or glomerulonephritis Bacteremia and remote metastatic infections occur only rarely, and fatality due to the toxicity of the infection is likewise uncommon In the absence of antimicrobial therapy streptococcal pharyngitis is seldom a relapsing disease

It is generally believed that rheumatic fever is a consequence of a perverted reaction of the host to its own products formed in response to the stimulus of streptococcal infection It appears that an increase in antistreptococcal antibody titer reflects the presence of the setting from which rheumatic fever arises in persons who are susceptible for reasons which are unclear Thus it was reasonable to attempt to suppress the development of these antibodies by the antimicrobial therapy of streptococcal pharyngitis with the notion that the antibodies either play an intrinsic role in the pathogenesis of rheumatic fever or that in their suppression the operation of hitherto unrecognized mechanisms is also suppressed Moreover this goal seemed to be desirable even if attained by sacrificing a possible valuable influence of the antibodies in the prevention of relapse of the pharyngitis

With the introduction of sulfonamide therapy it was possible to prevent the septic complications of streptococcal pharyngitis to such an extent that mastoiditis for example became a rare disease Moreover relapse of the pharyngitis after cessation of therapy was infrequent However it was soon demonstrated that the development of rheumatic fever could not be prevented by sulfonamide therapy even when treatment was started early in the course of the pharyngitis nor was the appearance of antibody materially affected

The introduction of penicillin therapy changed the situation in two important particulars On the one hand relapse of the pharyngitis appeared not infrequently after the cessation of therapy On the other hand the development of the manifestations of rheumatic fever could be virtually eliminated (and antibody formation markedly suppressed) if certain time dose relationships of penicillin therapy were maintained (Kilbourne and Loge 1948 Rammelkamp et al 1952)

In the Kilbourne Loge studies on a dosage regimen by which 'effective concentrations of penicillin were maintained for approximately 4 to 5 hours each day the results of the penicillin therapy of streptococcal pharyngitis were essentially the same as with sulfonamide therapy Septic complications were prevented but rheumatic fever occurred in some instances, and the incidence of patients showing a significant increase in antibody was only slightly less than in untreated patients In contrast, when a penicillin regimen was used which provided effective concentrations for the greater part of each day, the incidence of persons who showed an increase in antibody was sharply reduced, and the development of the characteristic manifestations of rheumatic fever was prevented

Thus, by the proper manipulation of the time dose relationships of a drug it is possible (in the specific circumstances described) either to suppress the infection without materially affecting the host reaction to that infection or to suppress both the infection and certain responses of the host to its presence

Cultural studies made on the two types of penicillin regimen revealed that the streptococci could not be isolated from patients on the continuous therapy whereas they could be isolated though not uniformly from patients on 'intermittent' therapy The latter situation is comparable with the results of cultural studies of patients treated with sulfonamide It is presumed therefore that the suppression of antibody formation on 'continuous penicillin therapy reflects a more drastic reduction in the size of the bacterial population and hence in antigenic stimulus than occurs with the other two less intensive forms of therapy

This rapid reduction in the size of the stimulus provided by the bacterial population carries with it an important disadvantage with respect to relapse In both its natural course and when treated with sulfonamide streptococcal pharyngitis usually persists for only a few days to a week However with the administration of penicillin for periods of 3 to 5 days even when effective concentrations are maintained continuously this relatively benign self limited disease may be transformed into an illness with 2 to 3 acute episodes in a period of 3 to 4 weeks So far as is known each of the episodes carries with it the same risks of the development of the suppurative or non suppurative complications as is the case with the original illness This happening can be prevented by sufficient prolongation of the penicillin therapy In actual practice it appears that 1 week may be too short to accomplish the purpose and that a satisfactory penicillin regimen consists of at least 10 days of therapy

The chemotherapy of infectious syphilis represents another situation in which the immune reactions to a particular infection develop differently depending upon the drug used for the treatment of the infection During the spirochetemia which occurs soon after the onset of infection the central nervous system is invaded by the micro organisms Despite this fact the development of clinically recognizable acute syphilitic meningitis in the absence of treatment is extremely rare When the early infection is modified by a few weekly injections of an appropriate arsenical drug followed by a *treatment free interval* the development of acute syphilitic meningitis is not uncommon In contrast with this situation the relapses which follow the inadequate penicillin therapy of early syphilis seldom if ever assume the form of acute syphilitic meningitis (Relapse with cutaneous and mucosal lesions may occur following short periods of therapy with either drug) Thus in the natural course of infectious syphilis or following penicillin therapy insufficient to prevent relapse elsewhere acute syphilitic meningitis is a most unusual event yet following inadequate arsenotherapy it is by no means unusual

The explanation for this different behavior of the infection under the two forms of therapy is not clear Although neither drug is transferred across the blood brain barrier in high concentration both are highly effective in the treatment of acute syphilitic meningitis when administered by systemic routes It is probable that the fact that penicillin has the greater antispirochetal activity of the two drugs is in some way responsible for the phenomenon

Another phenomenon first evident in the antimicrobial therapy of syphilis has since been observed in several other microbial infections namely that it may be possible to treat an infection *too early* in terms of attainment of the maximal antimicrobial effect The most satisfactory long term results obtained from the arsenotherapy of early syphilis occurred not when the treatment was started in the earliest stages but in those infections which had progressed to the point of overt cutaneous and mucosal lesions before the initiation of therapy A more striking example of a similar phenomenon is provided by the studies of Smadel Woodward and their associates (1949) on the antimicrobial therapy of scrub typhus

These investigators found that when scrub typhus proceeds naturally to the stage of an acute clinical illness only 24 hours of appropriate antimicrobial therapy (chloramphenicol or a tetracycline drug) are necessary to produce a complete disappearance of all evidences of illness Even if no chemotherapy is administered subsequently relapse does not occur at least during the immediately ensuing years * Moreover these excellent therapeutic results are obtained uniformly no matter how early in the course of infection chemotherapy is started, provided only that the infection has evolved to the point of clinical recognition In contrast when the same naturally acquired rickettsial infection in volunteers is prevented from evolving to the point of clinical illness by chemotherapy relapse follows cessation of the therapy even though the latter may have been administered for periods of several weeks Thus 24 hours of antimicrobial therapy are effective in the presence of clinical illness, whereas 7 to 21 days of identical therapy are ineffective when started before clinical illness has appeared

In studies with another rickettsial infection Q fever this phenomenon has been further defined by Tigertt and Benenson (1956) In their studies it was established first that 5 days of tetracycline therapy represented a completely satisfactory therapy for a clinically recognizable case of Q fever It was then shown that the incubation period of experimentally induced Q fever in man or in the guinea pig could be made predictable by appropriate manipulation of the dosage of infective particles In this standardized experimental situation it was found that with an infection with a predicted incubation period of 12 to 14 days the outcome was not affected substantially by 5 days of tetracycline therapy started immediately after the infection In some cases to be sure the incubation period was prolonged for a few days beyond the period predicted but in all cases the subjects developed the complete clinical illness of Q fever Up to this point therefore the results were the same as those previously observed experimentally with scrub typhus and inferred from the clinical experience with early syphilis However new information of considerable significance was provided by these Q fever studies For it was observed that if the 5 days of tetracycline therapy were given late in the incubation period immediately before the anticipated outbreak of the clinical illness no clinical illness developed Thus it was clear that for a satis

* The possibility that drug treated typhus may relapse many years later in a manner analogous to Brill s Disease obviously cannot be excluded at the present time

factory outcome from chemotherapy it was necessary for the infection to evolve to a certain stage, but that stage was present before the actual period of clinical illness

A related type of clinical observation was made in a small group of infants who had an exposure to tuberculous infection sharply limited to the first 1 or 2 days of life and were treated with isoniazid therapy thereafter (Lelong et al 1954) When the chemotherapy was started the infants showed no evidence of tuberculous infection, and their cutaneous reactions to tuberculin were negative During several months of isoniazid administration the infants developed normally and continued to show negative reactions to tuberculin However, within 4 to 6 weeks of cessation of isoniazid and without any known fresh exposure to tuberculosis, all 4 infants became tuberculin reactors Thus it appears that the isoniazid was able to hold these very early tuberculous *infections suppressed at a level so* low that cutaneous sensitivity to tuberculin failed to develop This illustration with tuberculosis is not so complete as the other examples because it has not yet been shown that there is another stage of the tuberculous infec*tion in which the end result of chemotherapy* is superior Nevertheless the isoniazid observations do show that despite what might be conceived as a most favorable situation from the standpoint of drug effectiveness the isoniazid was incapable of eliminating the infection

In vivax malaria, the existence of a certain degree of acquired immunity which is independent of persistent latent infection has been demonstrated by Coggeshall et al (1948) However, the degree of immunity observed does not seem to depend upon the length of time the infection had been present before it was treated but rather on the number of clinical relapses experienced by the patient Virtually no evidence of immunity was found in persons whose original infection had been treated successfully before they had experienced at least 3 clinical relapses

It appears therefore, that allowing the host parasite relationship to progress to the point of clinical illness or just before that point in certain infections provides a situation most favorable to the action of antimicrobial therapy

In the situations cited it is not known whether the obviously favorable conditions which have become operative by the time overt illness has appeared *represent a change* in the character of an important number of the parasites or the mobilization of immune forces of the host It is known, however that in these circumstances highly effective host forces of immunity do come into operation

The inferences generally made from these observations at present are as follows The mobilization of host immunity in the particular situations described (tuberculosis excluded) is necessary for successful antimicrobial therapy Suppression of the microbial populations *before* the mobilization of host immunity prevents the development of a stimulus sufficient to evoke the immune mecha*nisms and may induce or favor the* continuance of states of microbial persistence Suppression of the microbial population once clinical illness has appeared, which is presumably *after* the mobilization of host immunity has started, results in definitive control of the infection though not necessarily in elimination of all of the microbes

Thus, it is possible in at least certain instances to modify the response of the host to infection by the administration of certain antimicrobial drugs Apparently the effect on the host is an indirect one and results from reducing or otherwise altering the stimulus to the host ordinarily provided by a naturally evolving infection Likewise a certain neces*sary degree of evolution of the microbial com*ponent of the host parasite reaction may be prevented by the too early therapy

Therefore in order for a drug to have an inhibitory influence on host immune reactions the drug must have sufficient intrinsic antimicrobial activity and be administered with sufficient intensiveness to effect a change in the microbial population before the latter has reached the stage of serving as an adequate stimulus to the host In clinical practice the *presence of infection is not usually detected* until the infection has progressed to the point of producing clinical illness As a consequence in most clinical situations it is likely that a sufficient stimulus for host immunity has already been provided by the time antimicrobial therapy is initiated In a few situations however notably in streptococcal infections and possibly also in tuberculous infections manifested by the first appearance of tuberculin hypersensitivity the patient may receive med*ical attention sufficiently soon after the onset*

of infection so that appropriate antimicrobial therapy could exert an influence on the stimulus to the host Whether presently imperceptible diminutions occur in the degree of immunity mobilized by the host in many other infections treated with antimicrobial therapy is not known The possibility is a real one however and always should be considered in attempting to ensure that a sufficiently long total period of therapy is made available to an individual patient Finally though in general it is desirable that antimicrobial therapy be administered so as to interfere the least with the mobilization of immune forces there are situations such as the streptococcal rheumatic fever phenomenon in which it appears to be highly desirable to prevent the evocation of certain of the responses of the host

Chemoprophylaxis

The sequence of events that occur when an antimicrobial drug is employed very soon after the actual onset of infection has obvious relevance to considerations of the chemoprophylaxis of infectious diseases A prophylactic effort can be directed against any one of 3 different phases of an infection the actual initiation of the infection a stage immediately after onset and before evolution to the point of clinical recognition and the stage of a fully established infection with a potential for further progression to more serious complications Examples of the last named stage are the use of penicillin in streptococcal pharyngitis to reduce the chances of rheumatic fever or the use of isoniazid in young children who are cutaneous reactors to tuberculin Used in this stage of infection there is convincing evidence that appropriate drug administration does diminish appreciably the risk of serious complication It is not clear whether such a drug treated subject is generally left in a state less resistant to a renewed exposure to the infective agent than would have been the case had the same result been attained by natural means In all probability this is a matter that would be different with different infectious diseases It seems quite clear if the above cited experiences have generality that the administration of a drug *before* the microbe host reaction has matured to a certain stage imply results in an armistice between parasite and host which is soon broken once drug administration is discontinued On the basis of the experience thus far with this type of situation the ability of the host to cope with the infection once drug administration is stopped is no better than that of an initially infected host The clinician is seldom confronted with an infection in this phase Nevertheless the situation does arise occasionally notably in the form of a laboratory worker who has received an accidental puncture wound from a needle containing infective material

Prophylaxis aimed at the first phase namely the actual initiation of infection may likewise not always be successful because of the ability of most drug susceptible microbial species to survive in the tissues despite appropriate chemotherapy To be sure the actual physical transfer of microbes to a new host is presumably not always followed by their successful colonization there In this situation the further presence of an antimicrobial drug in the environment might well make the difference between the success or the failure of the implantation However the possibility is strong that even if the appropriate antimicrobial drug is present in the tissue environment at the moment of implantation certain of the microbes may survive as persisters The military experience with the administration of penicillin before exposure to gonorrhea and syphilis has bearing on this point Instances were observed of the development of syphilis despite the presence of penicillin in the subject at the time of sexual relations whereas gonorrhea was apparently prevented It is possible therefore that chemoprophylaxis directed against the actual initiation of infection will only be uniformly successful against the few microbial species such as gonococci meningococci and dysentery bacilli have very little capacity to display the phenomenon of microbial persistence

Interference With Mixed Microbial Populations

A subject of conceivable importance is the implications of the suppression of certain specise of a total microbial flora with emergence to predominance of other species For example with the administration of streptomycin chloramphenicol or a tetracycline drug the normal coliform members of the intestinal flora are suppressed with resulting overgrowth

of Proteus and Pseudomonas Likewise the nasopharyngeal flora and that of the sputum may be affected by the administration of penicillin so that only *E coli* and similar penicillin resistant species can be isolated There is some reason to believe that certain antimicrobial drugs notably the tetracyclines, exert an effect on amoebic infections not by direct action on the amoeba but by producing drastic alterations in the enteric bacterial flora It is not yet established whether these various alterations of the normal microbial relationships actually lead to enhancement of the pathogenicity of the surviving microbial species or whether the latter merely colonize a morphologic situation of such a nature that infection of one sort or another is inevitable

For example, in a patient with a chronically diseased urinary tract with renal calculi and various anatomic distortions infection is virtually inevitable When such an infection is apparently caused by *E coli* and is treated with a tetracycline *E coli* will disappear promptly and the infection will be controlled temporarily Soon thereafter however in some instances a new clinical illness may reappear in the form of a proteus infection Did the proteus microbes merely expand to occupy an abandoned dwelling so to speak or was their chance to produce disease in that host positively enhanced by removal of *E coli*?

The question of interference with the customary interspecies microbial relationships also becomes of importance in consideration of chemoprophylaxis especially the attempts to prevent the development of bacterial endocarditis following a surgical procedure or childbirth In the presence of an anatomic abnormality which tends to favor the development of infection is it advisable to attempt to prevent the development of infection by the administration of an antimicrobial drug during the period of anticipated greatest risk e g tooth extraction with a damaged heart valve? Or is it preferable to do nothing lest action result in interference with microbial relationships to the point that infection when it does occur is with a drug resistant organism? Definite information is lacking as to the answers to these questions at present In the absence of such information it seems to be advisable to pursue a middle course as much as possible and to use chemoprophylaxis sparingly only when the dangers of endocarditis seem to be reasonably great and immediate The same course is advisable when faced with the possibility of postoperative wound infections pneumonia, and other problems connected with surgical procedures

Whether the nutrition of man is significantly altered as a consequence of alterations in microbial metabolism within the gut or the nasopharynx also cannot be answered at present but it is clear that the nutrition of certain other animals can be affected dramatically In man some products of microbial activity the formation of urobilinogen, for example are suppressed markedly by the administration of certain antimicrobial drugs It is not clear however whether any of the metabolites synthesized by the bacteria of the gut of man are in fact utilized for the nutrition of the host As with chemoprophylaxis in the absence of information the sensible course would seem to recognize the possibility that such nutritional deficiencies may exist and avoid artificial suppressions of intestinal flora insofar as is possible

Local Versus Systemic Antimicrobial Therapy

In most instances in which antimicrobial drugs are used locally an accumulation of necrotic tissue is present As discussed previously such an area of necrosis provides the precise set of conditions least favorable for the maximal action of antimicrobial drugs or indeed for certain of the defenses of the host Such conditions are usually corrected by drainage and debridement simultaneously with the local application of the antimicrobial drug Moreover, the drug may be absorbed systemically to a considerable degree from the site of local application The simultaneous operation of these factors usually makes it very difficult to evaluate the precise degree of effectiveness of the "local administration of the available antimicrobial drugs In a particular instance it is usually impossible to judge to what extent a satisfactory therapeutic result represents the effects of the drug administered locally and to what extent the result reflects the consequences of the evacuation of pus and debridement As local therapy usually implies an accessible lesion it is probably more to the point to correct the unfavorable environment for both drug and host defenses by debridement and the evacuation of necrotic material than it is to ensure a high concentra

tion of drug in area Nevertheless there are situations in which local therapy might conceivably be of value

The principal situation in which local therapy might be of value is in certain instances of purulent meningitis in which it is difficult to attain sufficiently high concentrations of drug within the central nervous system solely on the basis of intramuscular administration In the case of penicillin which can be administered in virtually any dosage by systemic routes it has been possible to overcome this difficulty simply by marked increases in dosage With streptomycin however which can be administered only systemically in relatively low doses because of toxicity only minimal quantities can be transferred to the central nervous system when the drug is administered by the intramuscular route Whether or not these minimal quantities of streptomycin in the fluids of the nervous system represent the maximally effective concentrations for the treatment of certain forms of meningitis is debatable Certainly in most instances it seems to be necessary to add the intrathecal administration of streptomycin to effect a therapeutically satisfactory result As the other antimicrobial drugs now used for the treatment of meningitis are all transferred to the extracellular fluid of the central nervous system easily (or by enormous systemic doses as with penicillin) streptomycin represents about the only instance at present in which the intrathecal administration of a drug is necessary

Occasionally in meningitis or infection of some other body compartment the infecting microbe may be one which is susceptible only to concentrations of drug seldom attainable in the plasma or the extracellular fluid but readily attainable within an enclosed space by local administration An example of this type of situation is provided by enterococcal meningitis instances of which have been treated successfully by the intrathecal administration of penicillin It is conceivable that likewise certain localized staphylococcal infections might be treated successfully by the very large concentrations of drug attainable for short periods of time on local administration

Probably the greatest use of local therapy at the present time is in infections caused by *Pseudomonas aeruginosa* particularly meningitis empyema in burned tissues and infections of the urinary tract This micro organism is principally a scavenger of necrotic material and has a relatively low capacity to invade reasonably healthy mammalian tissue As a consequence in infections by Pseudomonas the removal of the necrotic material is of much greater importance than the use of a drug locally as can be seen by the experience of Tillett and Sherry with the use of the enzymes streptokinase and streptodornase These investigators have observed instances of empyema in which *Pseudomonas aeruginosa* was the predominating organism and in which all available antimicrobial therapy both systemically and locally failed to eliminate the microbes However following the administration of the enzymes the necrotic exudate was liquified and easily removed and the pleural cavities immediately became sterile In other words it did not appear to be possible to drive the invaders out by the administration of drug but they left promptly once the particular conditions necessary for their survival e g a large accumulation of necrotic material were no longer present

ANTIMICROBIAL AGENTS IN USE

General Considerations

In attempting to use antimicrobial drugs properly today the physician is confronted by a formidable dilemma On the one hand if therapy is to be maximally effective the correct drug or drug pairing must be chosen quickly so that it may be administered before significant tissue damage or irreversible physiologic changes have occurred On the other hand if the choice is to be made quickly it is difficult to make it correctly for there are very few microbiologic technics by which the cause of an infection can be positively identified during the early hours of acute illness

It is not always realized that there have been virtually no important diagnostic developments in the field of infections since the beginning of this century These 19th century technics were perfectly satisfactory when the principal purpose in identifying an infection was to obtain guidance in prediction of its course However the situation was sharply changed by the introduction of antimicrobial drugs for their chief role is to provide time for the patient to cope with the infection It is

of Proteus and Pseudomonas Likewise the nasopharyngeal flora and that of the sputum may be affected by the administration of penicillin so that only *E coli* and similar penicillin resistant species can be isolated There is some reason to believe that certain antimicrobial drugs notably the tetracyclines exert an effect on amoebic infections not by direct action on the amoeba but by producing drastic alterations in the enteric bacterial flora It is not yet established whether these various alterations of the normal microbial relationships actually lead to enhancement of the pathogenicity of the surviving microbial species or whether the latter merely colonize a morphologic situation of such a nature that infection of one sort or another is inevitable

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pains a few petechiae on the skin a systolic cardiac murmur and a leukocyte count of 15 000 cells per cu mm with an increase in immature neutrophils Two weeks previous to this abrupt onset of illness the patient had been exposed to a lyophilized culture of Brucella

It is obvious that the particular clinical syndrome presented by this patient could be produced by infection with a number of different agents Nevertheless unlike the first example in which *all* of the possibilities represented immediately serious threats to the patient in the present case only a few possibilities and really only one meningococcemia could be reasonably considered to represent an immediate threat to life Hence it was not necessary to treat the patient for all infections which might conceivably give rise to this type of illness but only for the one infection meningococcal, whose presence would constitute a serious and immediate threat Moreover the established therapy for meningococcemia sulfadiazine would have no effect on brucellosis or most of the other infections conceivably present Consequently if meningococcemia were not present the subsequent identification of brucellosis or bacterial endocarditis would not have been compromised

With the interpretation of the clinical data in this fashion the patient received sulfadiazine made an overnight recovery and blood cultures obtained prior to treatment eventually revealed the presence of meningococci

The 2 cases cited serve to illustrate the point that with the current multiplicity of drugs and the virtual absence of rapid diagnostic methods the initiation of proper antimicrobial therapy is a matter of microbiologic inferences drawn from the available clinical data The process becomes rational only in retrospect when the results of the tests started before treatment become available and permit appropriate modification of the therapy

The mere fact that an antimicrobial drug happens to have been developed later than another drug of proved value in a particular infection does not necessarily mean that its effectiveness is superior or even the equal of that of its predecessors Although it is a relatively easy matter to demonstrate that a newly introduced drug exerts substantial antimicrobial activity in a particular disease it may be very difficult to make any quantitative comparisons of the degree of its activity For example sulfapyridine penicillin and the chloramphenicol tetracycline group of drugs all exert an impressive effect on the course of pneumococcal pneumonia The degree of effect exerted by any one of these drugs is indistinguishable from that exerted by any of the others as observed in individual instances or in small series of patients Nevertheless it has been reasonably well established that penicillin is superior to sulfapyridine in terms of antipneumococcal effectiveness It is not always realized however that this superiority of penicillin is definitely perceptible only as a relatively slight reduction in an already small fraction of the patients treated This fraction consists principally of patients with unusually fulminating infections or those who are candidates for the development of serious metastatic complications such as meningitis As the area in which the comparative effectiveness of two antipneumococcal drugs can be measured is so small an enormous number of cases must be treated before a comparative evaluation is possible Thus with pneumococcal pneumonia which is by no means an isolated example only a small group of patients is necessary to demonstrate that a newly introduced drug has considerable activity Whether the degree of activity observed is comparable with that exerted by penicillin or more closely approximates that of sulfapyridine requires the careful study of hundreds of treated cases

The individual practitioner is able to avoid ethical conflict during the months or years necessary for the acquisition of such comparative data by limiting the use of a newly introduced drug to situations in which the infection does not constitute an immediate threat to the patient However in the early clinical trials of a new compound the physician is unavoidably faced with an ethical problem If he withholds drug A of known value for a trial of drug B of unproved value he may not be providing the most satisfactory antimicrobial therapy for his patient If he administers both drugs together he loses all opportunity for evaluation of drug B The device of making the initial trials of a new compound on some relatively harmless form of a particular disease provides no solution for it is in precisely such situations that the occurrence of a serious toxic reaction to the new compound could least be defended Unfortunately it is not possible in the present discussion to consider the available methods for meeting this problem It should be noted however that such an ethical problem exists and that the ways of solving it are becoming increasingly more difficult in view of the multiplicity of drugs of established value now available For a discussion of individual

precisely with respect to this factor of time that *the technologic methods for the identification of infections have become outmoded* In effect therefore a situation of stress has arisen whereby the physician has great power in the form of the available antimicrobial drugs and yet lacks almost completely the necessary knowledge and technics for the proper application of that power

The initiation of drug therapy without a diagnosis was a tolerable procedure when only one drug (sulfonamide) capable of exerting antimicrobial activity was available Even when the number of available drugs was 2 or 3 it was possible for the physician to compensate in some measure for his temporary ignorance of the etiology of a particular infection by administering all 2 or 3 drugs concurrently However with the relatively large number of drugs or drug combinations now available it is no longer feasible to follow this practice particularly as some of the drugs though highly active are limited in scope The physician is thus in the uncomfortable position of knowing that if he is to obtain the greatest advantages of antimicrobial therapy for his *patient he must make the correct choice of* drug or drugs 1 to 5 days before he can obtain solid evidence of the identity of the infection he is treating

An obvious way out of the physician s dilemma would be provided by the introduction of rapid diagnostic methods for the identification of infections Technics such as the fluorescent antibody detection technic of Coons (1955) might prove to be adaptable for widespread usage In the absence of such practical methods while passing through this period of imbalance between power and knowledge the most reasonable course is *to attempt to make* shrewd microbiologic inferences from phenomena which can be detected at the bedside Attempts should be made to differentiate on *clinical grounds* those situations or syndromes which require immediate and intensive action from those which properly may be left to unfold until precise identification becomes possible Moreover when a choice of therapy is made it should be made on the basis of careful consideration of the most serious threats to the patient which represent reasonable possibilities in the particular situation under scrutiny The drug or drugs chosen *should be the* ones which would do the most to protect the patient against the reasonably likely threats, while at the same time they would do the least to mask the identity of other infections that might conceivably be present

An example of the type of clinical practice involved is as follows A young man was first seen with a high fever, a cough and obviously excruciating pleural pain His systolic blood pressure was only 90 mm of mercury his leukocyte count was only 4 000 per cu mm, with a marked increase in immature neutrophils Less than 4 hours previously he had been enjoying himself at the theater though he had had a mild respiratory infection for the preceding 5 or 6 days

It was apparent that he had an infection that it was progressing rapidly, and that it involved lung and pleura What reasonable inferences could be drawn concerning the identity of the infection and hence both its relative threat to him and the appropriate choice of therapy?

Four microbial species *Pneumococcus Streptococcus M aureus* and *Klebsiella* (Friedlander s bacillus) are capable of producing this particular situation and there is absolutely no way of differentiating one from another at the bedside Nevertheless the clinician faced with this syndrome knows that it is apt to occur in such a severe fulminating form in less than 5 per cent of pneumococcal pneumonias whereas it is the characteristic picture of the rapidly necrotizing pneumonias caused by *Klebsiella Streptococcus* or *M aureus* Armed with this knowledge he knows that antimicrobial therapy must be started immediately Moreover *the choice of drug or drugs must provide maximal protection against Pneumococcus* and *Streptococcus* (penicillin) maximal protection against *Klebsiella* (streptomycin) and maximal protection against *M aureus* (penicillin and streptomycin with the addition of erythromycin if the presence of streptomycin resistant staphylococci is a reasonable probability) From 18 to 24 hours later when the identity of the infection becomes known the therapy can be modified appropriately

In the particular case described the cause of the illness was discovered to be a penicillin susceptible strain of *M aureus* which was controlled by penicillin

In the second case a laboratory worker was seen with an acute febrile illness of only 6 hours duration The illness was characterized *by chills a single episode of vomiting leg*

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Principles of Epidemiology

DEFINITION

There are three principal ways of advancing the understanding and the control of infectious diseases One is by studying each disease as it occurs in *individuals* and its causative agent (clinical pathology) The second is by studying each disease as its manifestations can be reproduced in *experimental animals* by the causative agent or its products (experimental pathology) The third is by studying each disease and its causative agent as it occurs among human animal bird and arthropod *populations in their natural environment* (epidemiology) These three approaches are overlapping and complementary Each borrows from the other but each has its own appropriate methodology This chapter is devoted to a discussion of the last named science—epidemiology

By derivation this science would seem to be concerned with the explanation of epidemics and indeed it began with the effort to offer a reasonable explanation of these striking natural phenomena This effort is as old as medical history As far back as 300 B C Hippocrates recognized that there were two kinds of epidemics Some forms of illness were not common and were not usually present but became epidemic in certain years and seasons and some forms of illness were always epidemic to a greater or less extent To this latter type of prevalence Galen later gave the name endemic For many centuries medical writers were content to describe the manner in which specific diseases prevailed as they were identified clinically from time to time in different localities in different climates and seasons among certain groups of people as compared with others and association with circumstances and conditions which seem to be pertinent

One of the most comprehensive of these collections is that of August Hirsch whose first edition of *Geographical and Historical Pathology* appeared in 1860 1864 (Hirsch 1883) As some medical men were trying to reason from the clinical manifestations in patients to the underlying pathogenesis so others were trying to reason from the mass phenomena of disease to the nature of its causation or its mode of communication Most of these arguments were highly speculative On the contrary a few were outstanding in their formulation of a sound hypothesis orderly array of observations and their logical inductive reasoning to justified inferences Such for example were the contributions of Oliver Wendell Holmes in 1843 on the Contagiousness of Puerperal Fever of John Snow (1865) on The Mode of Communication of Cholera and of William Budd in 1873 on The Nature Mode of Spread and Prevention of Typhoid Fever Their evidence pointed to the existence of microbial agents of disease long before experimental proof was offered by the work of Pasteur and Koch They demonstrated that this approach if pursued by objective scientific methodology had much to

chemotherapeutic agents see the last section of Chapter 33

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DEFINITION

There are three principal ways of advancing the understanding and the control of infectious diseases One is by *studying each disease as it* occurs in *individuals* and its causative agent (clinical pathology) The second is by studying each disease as its manifestations can be reproduced in *experimental animals* by the causative agent or its products (experimental pathology) The third is by studying each disease and its causative agent as it occurs among human animal bird and arthropod *populations in their natural environment* (epidemiology) These three approaches are overlapping and complementary Each borrows from the other but each has its own appropriate methodology This chapter is devoted to a discussion of the last named science—epidemiology

By derivation this science would seem to be concerned with the explanation of epidemics and indeed it began with the effort to offer a reasonable explanation of these striking natural phenomena This effort is as old as medical history As far back as 300 B C Hippocrates recognized that there were two kinds of epidemics Some forms of illness were not common and were not usually present but became epidemic in certain years and seasons and some forms of illness were always epidemic to a greater or less extent To this latter type of prevalence Galen later gave the name ' endemic For many centuries medical writers were content to describe the manner in which specific diseases prevailed as they were identified clinically from time to time in different localities in different climates and seasons among certain groups of people as compared with others and association with circumstances and conditions which seem to be pertinent

One of the most comprehensive of these collections is that of August Hirsch whose first edition of *Geographical and Historical Pathology* appeared in 1860 1864 (Hirsch 1883) As some medical men were trying to reason from the clinical manifestations in patients to the underlying pathogenesis so others were trying to reason from the mass phenomena of disease to the nature of its causation or its mode of communication Most of these arguments were highly speculative On the contrary a few were outstanding in their formulation of a sound hypothesis orderly array of observations and their logical inductive reasoning to justified inferences Such for example were the contributions of Oliver *Wendell Holmes in 1843 on the Contagious*ness of Puerperal Fever of John Snow (1865) on The Mode of Communication of Cholera and of William Budd in 1873 on The Nature Mode of Spread and Prevention of Typhoid Fever Their evidence pointed to the existence of microbial agents of disease long before experimental proof was offered by the work of Pasteur and Koch They demonstrated that this approach if pursued by objective scientific methodology, had much to

chemotherapeutic agents see the last section of Chapter 33

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HUMAN HOST RELATIONSHIPS

Clinical medicine is concerned with the reaction between the human host and the microparasite when it results in manifestations sufficiently marked to be regarded as an illness. Moreover it is centered upon one phase of the relationship, i.e. from the time of the onset of symptoms to recovery or death. On the other hand epidemiology is concerned with the whole relationship from the time the microparasites enter the body until they are eliminated. Interest is extended to include the conditions under which transmission of the infection takes place, the period of time which thereafter elapses before the appearance of symptoms, the occurrence of reactions below the threshold of clinical recognition, and the duration of the infectious state. All of these phases have an important bearing upon understanding the behavior of infectious diseases in human populations.

MODES OF TRANSMISSION

Knowledge of clinical pathogenesis, particularly when microbiologic methods are available for the recovery and the identification of the causative agent, affords information regarding the probable portal of entry and routes of exit of the microparasite in secretions and excretions. However, inferences drawn from observation on the conditions and the circumstances under which human infections occur in nature are commonly required to establish the route of communication or, at least, to suggest hypotheses to be tested by laboratory experiment.

For purposes of prevention it is useful to divide the contagious diseases into (1) those spread largely by secretions from the nose and the mouth (respiratory infections), (2) those spread largely through fecal discharges (alimentary infections), (3) those spread by genital contact (venereal infections), and (4) a residual group unclassified as to principal mode of spread. Accordingly, when reference is made to the spread of a disease from person to person by contact, it is desirable so far as possible to define further the meaning of this term.

This field of interest of epidemiology may be illustrated by reference to the mode of transmission of respiratory infections. Four mechanisms may be distinguished. Secretions from the nose and the mouth containing microparasites may be transferred (1) by immediate oral contact as in kissing or by contaminated hands, toys, instruments, or other material objects (fomites), (2) by large droplets expelled in coughing, sneezing, and talking and projected directly onto the conjunctiva or the oral region of another individual, (3) by smaller droplets (droplet nuclei) which according to particle size and weight may remain suspended in the air currents for a variable time period, (4) by dust particles originating from contaminated clothes, bedding, bandages, etc. The first two can be considered together conveniently as forms of personal contact. Opportunities for transfer by these means exist wherever human beings are associated indoors and outdoors. The two latter may be included properly in the term air-borne infection. It is a reasonable assumption that the risk of transfer by this mechanism is increased to the extent that human beings share a common atmosphere indoors. Furthermore, recent work has indicated that the size of air-borne particles containing microparasites which enter the respiratory tract during inspiration has an important bearing upon the initiation and the outcome of infection (Sonkin, 1951). The larger particles are filtered out by the defense mechanisms of the upper respiratory tract. The smaller particles may pass through the larynx, the trachea, and the bronchi to reach and be retained in the alveoli. Susceptibility to infection with each microparasitic species varies at different levels of the respiratory tract.

With these considerations in mind it is evident that the relative importance of transmission by personal contact and by air-borne particles must vary with the disease and with the circumstances of exposure. The effectiveness of personal hygiene and of aseptic technics in reducing spread by contact is too well known to require discussion. The role played by dust in the dispersal of hemolytic streptococci in hospital wards, barracks, etc., has been abundantly demonstrated (Robertson, 1947). Attempts to reduce the transmission of common respiratory disease in enclosed spaces occupied by groups of people by ventilation and disinfection of air have yielded equivocal results (Langmuir, 1951). Practical limita-

offer in the advancement of knowledge and the prevention of contagious diseases. It became apparent that this methodology could be applied to diseases of unknown etiology, to disturbances of metabolism, growth, or other physiologic states. From a doctrine of epidemics it became a science of broader scope dealing with the mass phenomena of disease.

Stated more specifically, *epidemiology became that field of medical science which is concerned with the relationships of the various factors and conditions which determine the frequencies and the distributions of an infectious process, a disease, or a physiologic state in a community.* It seeks to advance rational conceptual schemes of the causation of various ills which afflict mankind medically speaking. To the extent that this knowledge is advanced and valid, it becomes possible for appropriate community agencies to take effective measures directed toward prevention and control or eradication. The field is a broad one. For present purposes, consideration of the principles and the methods of epidemiology can be developed with advantage if *limited to infectious diseases.*

BASIC CONCEPT

For the synthesis of knowledge in a scientific field, a basic conceptual scheme is needed. It was not until after the turn of the past century, with the advance in general biology, that it was realized *that infectious disease is a manifestation of parasitism.* Perception of this fundamental concept marked an important transition. Since then, epidemic phenomena *have been interpreted in biologic terms* as expressions of the eternal struggle of living organisms for food by predation and parasitism, for shelter, and for the opportunity to propagate their kind. The implications of this basic concept have been elaborated in the chapter on parasitism and disease.

NATURAL HOST RANGE

The infectious diseases of man have emerged from a continuing evolutionary process. As a result of centuries of host wanderings, mutation and selective adaptation, certain worms, protozoa, fungi, bacteria, rickettsia and viruses have been successful *in maintaining their propagation in the biologic orbit of man* and are responsible for some of his ills. Their potentialities range from those which only rarely and inadvertently invade his tissues and cause occasional or sporadic cases of a rare disease to those which are dependent upon human tissues for their continued propagation, sometimes giving rise to epidemics which decimate tribes or nations and change the course of history.

The first requisite of a rational explanation of the behavior of infectious diseases in the human population is to understand *to what extent man as a host bears responsibility for the continuous propagation of the causative microparasite or shares this responsibility with other species, animal, bird, or arthropod.*

This concept affords a useful division of infectious diseases into two groups. In the first group are those which are caused by microparasites which have been successful in their survival relationships with man (a single host species) and his environment, such as the pneumococci, the meningococci, the gonococci, the shigella, etc. They are propagated principally by chain transmission from individual to individual by some form of direct or indirect human contact. Hence they are the cause of contagious diseases. The second group includes those diseases due to specific microparasites which are not directly transmissible from man to man, or transmissible only occasionally under unusual circumstances. These *noncontagious* diseases are caused by microparasitic species which are unsuccessful in maintaining continuous relationships with the human host alone. Their survival depends upon the intermediation of an arthropod vector or contact transmission in some animal or bird species, or they have found conditions under which they can survive, multiply and propagate outside the human body, or some combination of these host relationships. Propa*gation of these noncontagious* human diseases involves more than one host species in transmission chains of varying complexities, to which reference will be made later. Obviously, conditions which determine the natural distribution of the first group involving the ecology of a single host are comparatively simple and much more easily understood than those of the latter group.

TABLE 56 AGE DISTRIBUTION OF MENINGOCOCCIC MENINGITIS SANTIAGO CHILE 1942

Age	Population	Cases		Deaths	
		Number	Attack Rate per 100 000 Population	Number	Case Fatality (Per Cent)
Under 1 year	38 060	309	812	102	33 0
1 4	127 486	770	604	190	24 7
5 9	122 814	612	498	77	12 6
10 14	112 902	541	479	31	5 7
15 19	146 019	384	263	32	8 3
20 24	147 671	245	166	17	6 9
25 34	234 581	355	151	36	10 1
35 44	159 635	215	135	32	14 9
45 54	106 333	93	87	14	15 1
55 64	56 800	34	60	10	29 4
65+	38 306	22	57	12	54 5
Unknown		6			

Adapted from Horowitz and Perroni Arch Int Med 74 365 Table 2 1944

be made of the virulence or pathogenicity exhibited by a particular microparasitic species in the same community at different times or in different communities the case fatality ratio must be made specific with regard to age and any other pertinent variables Such ratios may be modified to a considerable extent by nonspecific conditions which affect the host population such as starvation lack of proper medical care secondary invasion by other micro organisms etc

Another scale of measurement of pathogenicity or virulence is the *ratio of clinical to subclinical infections* For example an attack of pertussis confers a durable immunity against a subsequent attack In the average American city about 75 per cent (Collins 1929) of adults give a history of having had a clinically recognized attack of the disease and more than 95 per cent of adults are immune Thus it would appear that 1 out of every 5 or 6 adults has acquired immunity by a subclinical infection

A single attack of diphtheria likewise confers upon most individuals immunity against subsequent illness from the same cau e On the basis of data collected in the United States in 1929 (Collins 1929) about 10 per cent of adults gave a history of having had the disease Surveys conducted in typical communities at this time indicated that approximately 60 per cent of adults had acquired immunity to diphtheria as evidenced by a negative Schick reaction It appeared therefore that perhaps 5 out of 6 individuals had gained their immunity through subclinical attack The ratio of clinical to subclinical attacks is not fixed precisely It varies within a limited range for each infectious disease in relation to age race biologic qualities of prevalent strains of the specific microparasite and other factors (Frost 1928)

Recent studies have revealed that about 3 out of 4 infections with *Coccidioides immitis* are subclinical and are identified only by change in reaction of the skin to coccidioidin Only 20 to 35 per cent of infections are clinically recognizable The manifestations of the disease vary from mild symptoms rarely diagnosed to the full blown characteristic syndrome known as San Joaquin or Valley Fever with malaise chills fever pleural pain cough and headache lasting a week or more In an occasional case perhaps 1 in 500 the disease goes on to progressive dissemination (coccidioidal granuloma) with a case fatality of from 30 to 60 per cent A decade ago the latter was the only recognized form of the disease (Smith et al 1946)

INFECTIOUS STATE

As early as 1890 Escherich noted that the *infectious period* of diphtheria was nearly coincident with the clinical course but that diphtheria bacilli might persist in the throats

tions have been encountered in the utilization of ultraviolet irradiation and of triethylene glycol vapors The employment of devices to control air borne transmission of respiratory infections is still in the exploratory stage

INCUBATION PERIOD

The first stage in infection is the interval of time from primary invasion through skin or mucous membrane to onset of symptoms of the disease It may be a matter of hours days weeks or even months depending upon the peculiarities of each host parasite relation ship but for any particular disease its length is relatively constant and predictable However as with other measurable biologic attributes for each there is a range of variation about the mean (see Fig 114) Most frequency distributions of incubation periods bear a resemblance to the normal curve with slight skewness rising more rapidly on the short side of the mean As Sartwell (1950) points out the degree of variation in relation to the magnitude of the mean has a constant statistical pattern no matter whether the unit of time be hours days weeks or months The range of the incubation period is an important epidemiologic characteristic of each contagious disease To a large extent it determines the rapidity of spread in a community As will be pointed out later the time span involved also serves as a basis for determination as to whether or not an outbreak is due to dissemination by a common vehicle

EPIDEMIOLOGIC PATHOGENICITY

Once initiated the ultimate issue of an infectious process is determined by the balance between the devices of aggression of a specific species of microparasite and the mechanism of defense of the host species which have been discussed in the chapters on immunology This *host parasite interaction varies greatly both in severity and duration* A *case* is a host reaction of sufficiently characteristic intensity and duration to permit clinical diagnosis Reactions which are less intense and of shorter duration are called *abortive* or *suspected* cases, the manifestations being too indefinite or protean in nature to permit diagnosis except in association with frank cases When the subjective and the objective symptoms are so slight as to pass unnoticed the host is said to suffer an *inapparent infection* Infections which are below the threshold of clinical recognition are grouped together as *subclinical* Their existence and frequency can be demonstrated directly or indirectly by such procedures as recovery of the infecting microorganism from the host tissues (carrier surveys) as for example, nose and throat cultures for diphtheria bacilli change in the response of the skin to antigenic material, as in the tuberculin test (tuberculin surveys) or change in serologic reactions from low titer to high titer (serologic surveys), as in the serum agglutination inhibition test with the viruses of influenza It is at least theoretically possible that micro parasitic infection may occur without demonstrable reaction on the part of the host i e a symbiotic or saprophytic relationship but there is a difference of opinion as to whether the word infection should be used to describe such a condition

Results of tests of the *virulence or pathogenicity* of a given microscopic species performed on one species of animal cannot be applied without qualification to another species Hence the virulence or pathogenicity of a given microscopic species for the human population can be assessed only by observations on groups of human beings exposed under natural conditions To the extent that infection may result in a case with recovery or death pathogenicity is roughly indicated by the proportion of attacks which are fatal Stated in different words it is the ratio between cases and deaths (usually the percentage of cases which are fatal) or the *case fatality ratio* of a disease This ratio is crudely expressed on the basis of cases and deaths at all ages However it varies with age and each disease has a characteristic pattern The following tabulation shows the pattern for meningococcus meningitis in an epidemic which prevailed in Santiago Chile in 1942 (Table 56) The case fatality rate was high in infancy decreased throughout childhood to reach its lowest figure in the age group 10 to 14 and then gradually rose with advancing years to a high in old age An array of this kind is useful in qualifying prognosis in relation to age With each disease it points out the age groups upon which preventive and therapeutic effort must be concentrated if mortality is to be reduced If comparisons are to

indicated indirectly by the number of deaths cases or infected persons

1 Cause specific death rate or mortality rate

$$\frac{\left\{\begin{array}{l}\text{Number of deaths from a particular cause in specified time} \\ \text{period}\end{array}\right\}}{\left\{\begin{array}{l}\text{Average population present during the same time period}\end{array}\right\}} \times 100\,000$$

2 Case rate attack rate or morbidity rate

$$\frac{\left\{\begin{array}{l}\text{Number of cases of a particular disease developing during a speci-} \\ \text{fied time}\end{array}\right\}}{\left\{\begin{array}{l}\text{Average population present during the same time period}\end{array}\right\}} \times 1\,000$$

3 Prevalence ratio

$$\frac{\left\{\begin{array}{l}\text{Number of cases of a particular disease at a specified time}\end{array}\right\}}{\textit{Population present at that time}} \times 100$$

The multiplying factors shown are those most frequently in use other factors may be used if desired so long as they are always stated in presentation of the material The time period ordinarily used in calculating (1) and (2) is 1 year although at times it may be computed for a longer or shorter period Obviously each of the three expressions has its own implications Use will depend upon the questions to be answered and the availability of quantitative data

Basic to effective use in reasoning is an assessment of the approximate validity of the rate or ratio This requires that the accompanying text contain a clear statement of the universe of observation in time (when) in place or area (where) and in persons (among whom) source of population estimates the methods by which deaths cases or infections were discovered and recorded and a description of the clinical and laboratory criteria used in diagnosis and classification Soundness of inferences drawn from biostatistical material never can exceed the level of accuracy of the original data These rates and ratios are so far as possible made specific for race age sex for some other factor which is being studied or for any combination of these In general the more specifically the individuals in both the numerator and the denominator are described the greater the degree of accuracy in representation This is particularly necessary where two population groups are to be compared

A death rate (1) or a case rate (2) are indices of incidence i e the rate of occurrence of a phenomenon during a given time period To represent the shift in balance or changes in equilibrium between a microparasite and the host population the expression of choice is ordinarily a series of attack rates based upon cases by date of onset report discovery or admission to a clinic in successive days weeks months or years (see Fig 113) If interest is limited to frequency of occurrence of such cases and the population at risk is relatively stable during the period under consideration it is unnecessary to calculate the rates The time sequence of the number of cases reflects changes in the frequency with which the microparasite is spreading and gaining access to new susceptible individuals and accordingly the increase or decrease in microparasitic population This is illustrated by Table 59

A statement of incidence should take into account not only the number of new cases (numerator) but the total number of new individuals at risk (denominator) in each successive time period It makes a great difference whether it is a closed or an open universe i e whether the population is composed of the same or approximately the same individuals throughout the period of observation or whether the individuals in the population are changing through immigration and emigration For example in one Army camp an incidence of cerebrospinal meningitis 10 times greater than in another when expressed on the basis of cases per thousand strength per year may be due to the fact that in the latter the personnel is permanent while in the former it is periodically changing through the arrival of recruits and the departure of graduates from a course of training so that 10 times as many individuals are at risk of infection during the course of a year

A prevalence ratio (3) is a cross section of experience at any specified time For example by survey procedures information is obtained as to what proportion of individuals in a group at any particular time are harboring a specific infectious agent such as diphtheria bacilli meningococci dysentery bacilli typhoid bacilli or show evidence of having had

of patients during convalescence In 1892 Guttmann Rommelaere and Simonds noted that cholera vibrios might be recovered from the feces during convalescence Credit belongs to Koch for grasping the important fact that cases which could be clinically diagnosed were not alone responsible for the spread of con tagious diseases In his studies of cholera in Germany during the winter of 1892 and 1893, he noted that some cases were so mild that they escaped recognition and indeed could be detected only with the aid of bacteriologic in vestigation The term 'carrier thus includes two classes First there are those who are about to have or have already had a clinical attack they are designated as incubatory or precocious convalescent and chronic carriers Second there are those who are suf fering from a subclinical or asymptomatic in fection the so called healthy ' carriers For the sake of clearness in thinking this second class of carriers should be included in the designation "subclinical ' or ' inapparent ' in *fections*

The duration of the infectious state may be brief or protracted Cholera vibrios disappear from the dejecta of a patient usually within 5 to 7 days after onset and persistence up to 15 days is extremely rare chronic carriers are unknown Cultures of stools for *Shigella* or ganisms are usually negative after 4 or 5 weeks a few remain positive for several months and occasionally for years About 3 per cent of cases of typhoid fever become fecal carriers for the remainder of their lives

The *duration of the infectious state* in cases *and the frequency of inapparent infections are* factors of considerable importance in propaga tion and survival of a microparasitic species in relation to the human host The opportunities for scatter and chance of transmission (con tagiousness or communicability) are increased in proportion to the length of time the progeny can continue to multiply and find egress from a host particularly one who is ambulatory The longer the duration of the infectious period, and the greater the frequency of mild inapparent ambulatory infections the more difficult it becomes to decrease the spread of a disease by *such measures* as isolation of cases and quarantine of close associates

MICROPARASITIC SURVIVAL IN EXTERNAL ENVIRONMENT

Pathogenic fungi and bacteria exhibit a wide range of variation in their ability to *resist physical conditions in their external environ ment* such as sunlight, drying high and low temperatures etc At one end of the scale are the meningococci and the gonococci which die outside the body within a few minutes and at the other end of the scale are the anthrax *spores which may remain latent in soil for* years Differences in epidemiology of typhoid cholera and bacillary dysentery depend to a considerable extent upon differences in the ability of each species of micro organism to survive in the external environment and par ticularly in water

INCIDENCE AND PREVALENCE

The forces which create the dynamic bio logic phenomena of infectious diseases are in the ultimate analysis population pressures i e the innate impulse of living micro organ isms to multiply and survive by parasitism upon *homo sapiens* and intelligently directed efforts of the host species to preserve its own integrity The balance between these two forces is constantly fluctuating as are the interactions between other living species as for example between the carnivores and their herbivorous food sources When the equi librium is a relatively stable one it is mani fested by an "endemic ' prevalence When the equilibrium is subject to sudden and violent disturbances it is manifested by epidemics If the balance is in favor of the host *the dis ease shows a downward trend and tends to* disappear If the balance is in favor of the microparasite the disease tends to increase in prevalence and in certain instances may act as a human population check

To facilitate reasoning with regard to the infectious disease of man it is necessary to express these phenomena in quantitative terms (Densen 1951) For this purpose certain rates and ratios are conventionally used The basic elements of this statistical methodology are formulae which indicate the size of the parasitic population relative to the number of the human population at risk The first term cannot be measured directly, but it can be

(Commission on Acute Respiratory Diseases 1946) Others range from being rather constant in their endemic level as for example tuberculosis to being unpredictable as for example pneumococcus pneumonia

Each contagious disease has a seasonal variation which follows a more or less regular pattern reaching maximum distribution about the same time each calendar year when it can be assumed conditions are most favorable to transmission In general respiratory infections reach maximum incidence in the colder months of the year alimentary infections in the warmer months (Aycock et al 1945) Although there is much speculation little is known about the factors which determine this difference

Correlated with interannual and seasonal variation of each contagious disease is an apparent movement through populations and geographic areas With some a wavelike spread across states and regions can be distinguished as has been the case with outbreaks of meningococcic meningitis Others as for example typhoid fever remain more or less localized and focally distributed Since the conditions required for propagation involve only the ecology of the human host they may be encountered anywhere in the world A notable exception is cholera Apparently this disease can spread only in crowded communities of impoverished peoples living under primitive conditions of sanitation It is endemic in certain areas of southeastern Asia principally in some of the Indian states from which it extends epidemic pseudopods into the adjacent regions from time to time These extensions have become more and more restricted with advances in sanitary practice

Each contagious disease has its peculiar race sex and age selection The most important of these is age as indicated by observed cases or history of previous attack This selection is determined by conditions of exposure and the durability of immunity Where exposure is inevitable in the course of a few years and a single experience with infection confers a durable protection against subsequent attack it is a children s disease such as measles and chicken pox The longer exposure is delayed the more the age distribution shifts toward the older brackets as in meningococcic meningitis (see Table 56) Where infection confers only transient immunity as appears to

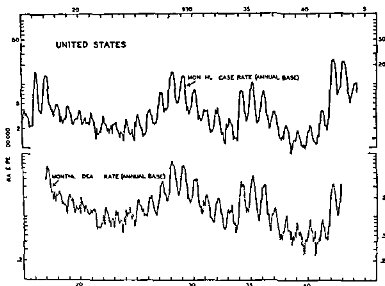


Fig 113 Monthly morbidity and mortality (annual base) from cerebrospinal meningitis in the United States from 1916 to 1944 Deaths are for cerebrospinal (meningococcus) meningitis (Gover M and Jackson G 1946 Cerebrospinal meningitis A chronological record of reported cases and deaths Pub Health Rep *61* 440)

a preceding infection of some type, as indicated by a positive serologic test for syphilis, a positive tuberculin test or a positive Schick test etc

Thus in a study of *meningococcal infections* in a military population Phair and Schoenbach (1944) made nasopharyngeal cultures from 99 men 3 times weekly over a period of 68 days The per cent found positive on each examination varied from 22 5 to 51 the *median prevalence ratio* for the whole series being 40 per cent This is a static concept It was desirable also to know the dynamics of the situation i e how many new individuals were being infected (or the *incidence* of new infections) in successive culture periods This would have indicated whether the microparasitic and the host populations were in equilibrium or whether the balance was temporarily swinging in favor of one or the other In summation of the experience during the whole study period it was found that of the 99 men 92 or 93 per cent had been culturally positive on one or more occasions

EPIDEMIOLOGIC INVESTIGATIONS

Understanding of the epidemiology of an infectious disease develops with the accumulation of observations and records of its incidence in different localities population groups and time periods in relation to what appear to be pertinent conditions circumstances and associated phenomena At successive stages in this advance attempt is made by inference and synthesis with current knowledge to formulate a rational hypothesis which will satisfactorily account for the behavior of the disease The undertaking may be a study concerned with information collected from a large geographic area a large population unit as of states regions or metropolitan areas armies, and cover decades or centuries in time—the natural history of a disease It may be concerned with information collected from a limited locality a small population unit such as a city town village neighborhood institution military post and over a short period of time days weeks or months i e the investigation of an epidemic It may be concerned with some combination of both The principles are the same The sources of information and the methods of analysis differ In the large scale study the data are derived from miscellaneous references in medical literature and the analysis of routinely collected morbidity and mortality reports In the epidemic investigation the data are obtained by an individual or group as a result of personal inquiry and by filling out an appropriate schedule of information on each case and death together with such special surveys as are desirable and feasible The former affords a general concept of the over all behavior of a disease The latter often affords opportunity to examine a deviation from this general pattern which has practical value from the standpoint of future prevention and not infrequently, an illuminating scientific value Since both the general pattern and the epidemic characteristics of contagious diseases are quite different from those of the noncontagious diseases they will be discussed separately

GENERAL PATTERN OF CONTAGIOUS DISEASES

The general features of a contagious disease are delineated by consideration of fluctuations in its incidence over months years, decades, its spread in relation to geography, and its selective distribution among different groups of people With regard to time each manifests its peculiar *interannual variation* or secular trend Years of relatively high prevalence are interspersed with years of relatively low prevalence

Figure 113 illustrates the manner in which the prevalence of meningococcic meningitis has varied in the United States during the period from 1916 to 1944 (Gover and Jackson 1946) The word epidemic may perhaps be used to describe the periods of high prevalence even though they are of several years duration It is apparent from the Figure that there have been 4 such periods reaching maximum rates in 1916 1929 1936 and 1943 The number of years included in each epidemic period is obviously a matter of arbitrary decision and depends upon the definition of excessive prevalence which is employed

In similar manner, the *interannual variation* in the numbers of cases of measles in Providence R I is shown in Table 59 Every second or third is a high (epidemic) year The intervals between epidemic years vary with each contagious disease and population unit some being rather regular and predictable

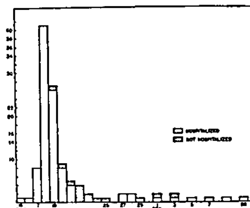


Fig 114 Distribution of cases of type 5 streptococcus infection by date of onset of symptoms (Commission on Acute Respiratory Diseases 1945 A Study of Food Borne Epidemic of Tonsillitis and Pharyngitis due to B Hemolytic Streptococcus Type 5 Bulletin of the Johns Hopkins Hospital 77 143 210)

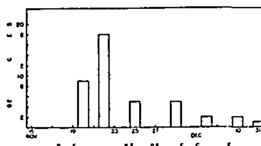


Fig 115 Distribution of healthy carriers of type 5 streptococci by date of first positive culture (Commission on Acute Respiratory Diseases 1945 A Study of Food Borne Epidemic of Tonsillitis and Pharyngitis due to B Hemolytic Streptococcus Type 5 Bulletin of the Johns Hopkins Hospital 77 143 210)

simultaneous exposure to a common medium of dissemination or to a single source Or reversing the procedure if it be known that the group of persons selected by a disease have been together upon only a single occasion then the common exposure must have occurred at this time and the variation in the incubation periods of different individuals can be calculated

These considerations may be illustrated by an epidemic of tonsillitis and pharyngitis due to β hemolytic streptococcus Type 5 in the members of two companies of an air borne infantry regiment at Fort Bragg The following account is paraphrased from the report of The Commission on Acute Respiratory Diseases Fort Bragg N C (1945)

The chronologic sequence of development of the epidemic is illustrated in Figures 114 and 115 the former indicates the date of onset of symptoms of hospitalized and nonhospitalized cases * the latter the date of discovery of healthy carriers †

* Nonhospitalized cases represented subjects who made dispensary visits for symptoms referable to the respiratory tract within a week after Type 5 Streptococci were first isolated in their throat cultures

† Editorially defined as subclinical or inapparent infections

Primary and Secondary Attack Rates The definitions of the terms primary and secondary were of necessity arbitrary but were made after consideration of all the facts elicited in regard to the time of original infection and incubation period Primary cases were defined as subjects who became ill and harbored Type 5 Streptococci at the time of the first culture survey on November 20 Figure 115 indicates that more than 75 per cent of the total number of cases had an onset of symptoms on or before that time Epidemiologic evidence dated the time of primary infection as the morning of November 17 Two cases had a clinical onset before November 17 this discrepancy may have been due to an error in history taking or to the presence of respiratory symptoms due to other causes antedating the infection with Type 5 Streptococci Secondary cases were defined as those who did not harbor Type 5 Streptococci on November 20 or before but subsequently became ill and acquired the organisms Early secondary cases were those who acquired the organisms before November 27 and late secondary cases those who first harbored the organisms after that date Healthy carriers were classified by the same cultural criteria as cases

Since 27 men had already become ill by noon of November 18 there were numerous foci of infection scattered throughout the group for 48 hours before the first culture survey was made It is therefore entirely possible that some of the cases and carriers classified as primary may in fact have been infected secondarily by contact in the barracks with earlier cases or carriers However the

be the case with influenza, the individual may be reinfected with the same microparasitic species after a short interval. *Consequently, the* disease may attack all ages—adults and old people as well as infants and children.

The age distribution of immunity as indicated by tests for the presence of specific antibodies on a representative sample of the population reflects the past experience of a *community with a particular microparasitic* species. Thus the simplest expression of the history of a community with regard to exposure to tuberculosis is the proportion of individuals found positive by a tuberculin test at successive ages and in various periods. Considerable insight into the natural history of *the virus of poliomyelitis, particularly the* Lansing type, has recently been obtained by tests for neutralizing antibody on sera from representative population groups according to age. A good example of the value of this kind of observation is the report of Paul and Riordan (1950) on their findings with regard to the Lansing strain of poliomyelitis virus in sera from Alaskan Eskimos. In the belief that Eskimos have less exposure to poliomyelitis than do most other populations, serum samples from Eskimos living on the north coast of Alaska were collected and studied. Particular attention was paid to the presence or the absence of neutralizing antibodies to the Lansing strain of poliomyelitis virus. Poliomyelitis is known to have been present along this north coast in 1930, but among the scanty and unreliable records available there is no note of its occurrence since that date. The neutralizing antibody test of this Lansing strain of poliomyelitis virus revealed that nearly all natives who were tested from the two villages and were below the age of 20 failed to have antibodies, whereas in those who were age 20 and above about 80 per cent had these antibodies in their sera. The evidence suggested that there had been no exposure to or infection with the Lansing strain in this remote community since 1930.

EPIDEMICS

While the term "epidemic" may be used to refer to a peak in the oscillating incidence of a disease, it is perhaps more commonly used to *designate a sudden or unusual disturbance* in the balance between the host and the microparasitic population. How great the increase in incidence must be before it is regarded as an *epidemic is a matter of judgment and is influ*enced by psychological attitudes. The greater the fear of a disease and the more unusual its occurrence in a community, the smaller the increase need be to justify the use of this descriptive term. Many statistical devices have been suggested for making the definition more *objective*, but none has received general sanction. Dependence is usually placed upon comparing the current incidence of a disease with its incidence in the past in the same population group and at the same time of the year. The expected number is commonly expressed as a 3 year or 5 year median of reported cases. When current incidence exceeds this number the disease shows a trend which, if sustained and great enough, merits being called an epidemic.

For investigative purposes, two kinds may be distinguished, namely, (1) common vehicle epidemics and (2) progressive or propagated epidemics.

COMMON VEHICLE EPIDEMICS

An unusual or unexpected incidence of a disease is conventionally represented in a graph by plotting the number of cases (ordinates) by date of report or onset according to the selected time intervals: hours, days, weeks (abscissa). The numbers usually show a regular ratio of increase in successive intervals to reach a maximum and pass over into a similar ratio of decrease so as to describe a more or less symmetrical curve, as illustrated in Figure 114.

It is to be noted that the span of time between the minimum and the maximum incubation periods varies widely in different diseases. For example, in food poisoning due to staphylococcus toxin it is a matter of from 1 to 8 hours, in influenza from 1 to 2 days, in measles from 12 to 16 days, in homologous *serum jaundice from 2 to 6 months*. By comparison of this span of time of the disease involved with the period during which the cases included in the outbreak have their onsets, an important inference can be drawn. If the onsets of all or nearly all the cases fall within an interval no greater than that of the known variation in incubation periods, then it can be assumed that they arose from a nearly

TABLE 58 RELATION OF PRESENCE OR ABSENCE OF TYPE 5 STREPTOCOCCI IN THROAT CULTURES ON NOVEMBER 20 TO HISTORY OF EATING SPECIFIED FOODS ON NOVEMBER 16 AND 17

	Total with Food History Known		Type 5 Streptococci				
			Number		Per Cent		
Food	Ate	Did Not Eat	Ate	Did Not Eat	Ate	Did Not Eat	P *
Spareribs	129	9	73	4	57	44	0.47
Fruit salad	127	10	72	5	57	50	0.69
Creamed eggs	63	62	50	22	79	35	0.00001
Cauliflower	43	85	27	46	63	54	0.35
Noodles	95	31	59	15	62	48	0.16

* Probability that differences as great as these could arise from sampling

amination or history of recent infection in any of them

The investigation of an explosive outbreak may be relatively simple since a priori one is concerned only with discovering the common factor A microparasitic population has suddenly found an opportunity and a medium by which it can be disseminated to a group of host individuals in a short space of time A certain proportion of the exposed group are susceptible and to that extent they come down with clinical or subclinical attacks characteristic of the specific infectious agent The problem is resolved into discovering upon what common occasion or by what common medium the persons so selected could have had a more or less simultaneous exposure

If the portal of entry of the specific micro-parasite involved is or may be through the alimentary tract attention is then centered upon articles of food or drink particularly water supply milk supply or food that has been insufficiently cooked or has been allowed to stand several hours after preparation in a warm place allowing opportunity for growth of the pathogenic micro organisms The remainder of the investigation is then directed toward elucidating the conditions which permitted the contamination to occur with the practical objective of instituting appropriate preventive measures

PROPAGATED EPIDEMICS OF CONTAGIOUS DISEASE

When the span of time of an epidemic wave is greater than the limits of variation in the incubation period of the particular disease in question it can be assumed (1) that the dissemination by a common vehicle medium has been prolonged as in some water borne outbreaks or (2) that the infection is being propagated by man to man chain transmission or contact or (3) that there is a combination of both If the circumstantial evidence is inconsistent with transmission by a common vehicle then some change must be sought in one or more of the factors which affect the rate of dissemination of the causative microparasite in the community involved For the common contagious diseases the principal factors to be considered can be enumerated

Theoretically at least increased dissemination of a contagious disease in a given locality at a particular time may be initiated by change in the biology of the invading species and type of microparasite The limits within which bacteria and fungi have the capacity to vary and mutate toward higher or lower communicability and virulence has received attention in a preceding chapter and need not be repeated here It suffices to state that some species are relatively stable others show considerable ability to vary In general with any given species those strains tend to propagate which are selectively best adapted to multiplication and survival in the human population at a particular time There is little or no actual evidence however that a change in the virulence or pathogenicity of a microparasitic species commonly initiates the upward swing in incidence which marks the beginning of an epidemic or the downward swing which leads

TABLE 57 PRIMARY AND SECONDARY ATTACK RATES IN 228 ENLISTED MEN IN G AND HQ COMPANIES

Type of Infection	Strength Exposed to Risk	Number			Per Cent		
		Cases	Carriers	Total	Cases	Carriers	Total
Primary	228	86	9	95	37 7	3 9	41 7
Secondary							
Early	133	11	13	24	8 3	9 8	18 0
Late	109	7	9	16	6 4	8 3	14 7
Total	228	104	31	135	45 6	13 6	59 2

separation made of primary and secondary cases and carriers seemed to be the most reasonable with the data available

Primary and secondary attack rates were determined on the group of 228 enlisted men in G and Hq Companies who were present on November 17 and remained under observation until December 31 During this period all hospital admissions were studied dispensary visits counted and 6 periodic culture surveys made The primary attack rate for this group was 41 7 per cent (Table 57) Of these 86 were primary cases and 9 carriers The secondary attack rate among those exposed to risk was 30 1 per cent of which somewhat less than one half were secondary cases and slightly more than one half were secondary carriers In the course of 6 weeks from the beginning of the outbreak 59 2 per cent of the men in the group acquired Type 5 Streptococci 45 6 per cent were cases and 13 6 per cent carriers

The case to carrier ratio of the primary infections was approximately 10 to 1 which suggested a massive dose of infection and indicated that almost all subjects who acquired the infectious agent at the time of the original seeding became clinically ill Among the secondary infections the case to carrier ratio was approximately 1 to 1 More than two thirds of the healthy carriers appeared to acquire the organisms as a result of secondary contact spread

Source of Infection Because of the explosive nature of the epidemic and the concentration of cases with onsets on November 18 and 19 a common source of infection was immediately suggested The further evidence of localization of the cases entirely to two companies served by a common mess and the absence of infection in an adjacent company served by a separate mess pointed strongly to food as the vehicle of infection From a consideration of the time of onset of illness and the probability of an incubation period of not more than 3 or 4 days the search for the source of infection was concentrated on foods served at mess on November 16 or 17

Investigation indicated that the milk supply could not have been infected unless it was contaminated after delivery to the mess hall The milk was pasteurized and delivered in quart bottles from a large dairy which regularly served more than 10 000 troops on the Post

On November 23 the enlisted personnel and officers were questioned regarding specific items of food eaten on November 16 and 17 Infection rates were calculated according to the history of eating specified foods the infected individuals being those who harbored Type 5 Streptococci on the cultural survey conducted on November 20 (Table 58) Infection rates among those who gave a definite history of eating or not eating the particular items of food revealed a significant difference only for creamed eggs which were served for breakfast on November 17 Seventy nine per cent of those who said they had eaten the eggs were infected, whereas only 35 per cent of those not eating eggs had positive cultures on November 20 The probability that this difference was the result of chance alone is 1 in 100 000 The source of infection of the 22 men who said they had not eaten creamed eggs could not be determined The dietary interviews were conducted 6 days after the creamed eggs were served and therefore were subject to considerable error It is also possible that some of these 22 cases may have been early secondary infections At the time the investigation was made none of the food served before November 20 was available for bacteriologic examinations The exact manner in which the creamed eggs were prepared was not ascertained because of conflicting testimony It was established however that the eggs had been boiled and then sliced by hand at least 10 hours before serving None of the cooks harbored Type 5 Streptococci on November 20 nor was evidence obtained by ex

one of the principal objectives of an epidemiologic investigation because upon such knowledge effective control depends

EPIDEMIC THEORY

From the epidemiologic point of view the simplest of all infectious diseases is measles Table 59 illustrates the manner in which its incidence varies in any large city By progressive host to host transfer the virus population maintains itself more or less continuously If it dies out completely before long it is reintroduced by the importation of a case in the infective stage However there is a rhythmic variation in incidence correlated with the season of the year increasing to a maximum in the pring and decreasing to a minimum in the summer months The time at which the maximum incidence is reached in each year varies within fairly wide limits In some years the total incidence is relatively low in others it rises to a level regarded as epidemic These epidemic years appear to recur at fairly regular intervals in the same locality

A century ago the periodicity of measles epidemics was known and discussed (Hirsch 1883) The causes were thought to be obscure and complex although it was generally accepted that the accumulation of susceptibles was an important factor A more precise numerical approach to the explanation of periodicity of measles began with the contribution of Sir William Hamer (1906) Following his lead a biometrician (Soper 1929) in the course of an examination of possible method of forecasting common contagious diseases was led to adopt the simplest mathematical postulate that would describe on a first measure the generally accepted mechanism of epi

TABLE 59 MEASLES CASES BY MONTHS IN PROVIDENCE R I FROM 1917 TO 1940*

YEAR	JAN	FEB	MAR	APR	MAY	JUNE	JULY	AUG	SEPT	OCT	NOV	DEC	TOTAL
1917	33	47	62	109	119	36	13	7	2	1	8	55	492
1918	55	98	3 3	1 232	1 299	780	261	23	8	6	5	3	4 143
1919	1	4	4	4	5	4	3	3	1	2	1	3	35
1920	125	127	136	279	404	288	146	38	45	53	190	191	2 0 2
1921	329	585	665	390	266	99	28	10	1	2	7	26	2 408
1922	89	4	3	26	25	22	23	19	7	16	131	652	1,017
1923	680	1,229	1 470	687	383	117	29	6	3	10	7	7	4 627
1924	5	6	3	11	16	30	15	2	2	1	5	2	98
1925	13	11	6	15	18	30	58	50	13	81	417	1,224	1 936
1926	2 057	1,360	648	348	196	105	48	8	1	0	0	4	4 775
1927	5	2	1	1	2	2	6	2	0	9	7	23	60
1928	45	112	422	1,081	883	900	508	77	18	36	36	61	4,0 9
1929	84	189	261	399	2 6	111	38	4	3	2	0	0	1,367
1930	2	0	1	4	23	46	22	8	1	0	2	0	109
1931	1	2	49	158	456	358	179	99	22	191	337	1 548	3 400
1932	2 99	2 037	574	199	81	11	2	0	0	0	0	0	5 03
1933	0	0	0	3	3	6	5	2	4	0	1	1	25
1934	4	11	21	18	29	106	44	25	8	5	1	7	279
1935	13	57	343	1,351	1 953	1 279	241	17	4	1	0	48	5,30
1936	119	74	92	76	83	17	11	4	0	0	9	77	562
1937	422	811	1 184	711	472	129	31	4	0	2	3	3	3 772
1938	2	5	4	2	0	0	0	3	1	0	0	3	20
1939	33	35	40	118	317	286	157	64	20	89	267	446	1 872
1940	569	495	530	462	543	372	121	20	1	0	1	1	3 115
Total	7 485	7,300	6,892	7 684	7 852	5 034	1 989	495	165	507	1 435	4,385	51 223

Epidemics culminate in May 1918 March 1921 March 1923 January 19 6 April 1928 January 1932 May 1935 March 1937 March(?) 1940 In this period of 262 months there are 9 major peaks but we must not count both ends The average time between peaks is 33 ± 7 9 months not 2 years For the mean we write 33 ± 2 8 months In Glasgow we estimate 40 months between peaks from 1888 to 1927 incl based on Sopers data [J Roy Statist Soc London 9 34 61 (1929)] How many peaks one cou t depends on the interpretation one gives to the qualifying adjective major and what allowance one makes for seasonal interruption of an epidemic

* Adapt d f om Wil on E B and Bu ke M H 1943 The epidemic curve II Proceedings of the National Academy of Sciences 9 43 48

to its termination Other factors appear to be of more importance

The human community is made up of a number of individuals who vary not only in their genetic capacity to react but nearly always in their previous experience with dominant strains of a particular species of microparasite or its close relatives Some individuals have acquired complete immunity some a partial immunity some none The proportion of a population at any one time which has little or no immunity determines the theoretical level of a community group or herd susceptibility for a particular contagious disease

The critical level of susceptibility ('epidemic potential') required for the rapid passage or epidemic spread of a contagious agent varies from community to community, and even in the same community, at different times depending upon population density and other factors The degree of crowding or dispersal of a population may be indicated in many ways the most common is the classification into urban and rural populations Quantitative estimates are sometimes made of the number of persons per square mile the number of individuals per house or the number of persons per room These expressions are intended to convey in a rough way an idea of the frequency with which one individual may meet or have contact with another individual under conditions which would permit the passage of infectious agents from one to another The association of the unusual prevalence of contagious diseases with crowding in communities institutions jails ships barracks refugee camps etc is a very old one

Opportunity for the dissemination of microparasites is obviously facilitated by the amount of population movement travel and flow of people through a community In isolated small communities with a small natural turnover in population by births and deaths many of the common communicable diseases are unable to maintain themselves over long periods of time because of acquired immunity they tend to die out and must be reintroduced Under modern conditions of travel however such communities are encountered only in the most remote parts of the world Today there is a constant flow of people into and out of most communities A sudden increase in the incidence of a disease may be precipitated by the arrival of a large number of susceptible individuals such as refugees immigrants or recruits

The customs and the habits of the people are obviously important in affording opportunities for transmission For respiratory diseases this relates to customs with regard to uncontrolled coughing spitting and sneezing For enteric infections it relates to the habits of fecal disposal hand washing etc For venereal diseases it relates to the habits with regard to promiscuous sexual intercourse The general concept is expressed in terms of the level of household cleanliness and personal hygiene In communal living the potentialities of common water milk and food supplies and of methods of sewage and refuse disposal for the dissemination of pathogenic microparasites are well known Indeed the downward trend on many of the contagious diseases particularly of the enteric group during the past half century is the result of sanitary measures designed to reduce to a minimum the stream of fecal micro organisms through the human alimentary tract by common vehicles

Temperature humidity rainfall and sunlight may influence the equilibrium between the microparasite and the host population in a number of different ways Such factors may directly influence survival of a microparasite in the external environment on objects in soil dust water and food Thus they may facilitate or retard the passage of the microparasite from one host to the next On the other hand these factors may affect the resistance or the immunity of the host population Less is known about the latter effect but there is some reason to believe that resistance to certain microparasites may be affected indirectly by changes which take place with season and more particularly with sudden extreme changes in body temperature

It is apparent from this brief summary that the host parasite balance is determined by large numbers of factors and conditions and is the resultant of a complex biologic situation While to a considerable degree these factors and conditions may be recognized described and enumerated their relative importance varies with each contagious disease and with local circumstances To assess their relative importance for a particular disease in relation to a definite locality population and time is

operation of chance In the community where sufficient data are available the correspondence between cases predicted by such a formula and the cases observed is reasonably good within certain limits The same kind of reasoning and mathematical postulations can be applied to other contagious diseases but the factors which must go into the equation become more complex and we are unable to obtain numerical values for them from observations made in nature The practical usefulness of the statistical approach to epidemic theory becomes correspondingly limited

EXPERIMENTAL EPIDEMIOLOGY

Another approach to the discovery of laws or general principles governing the behavior of infectious diseases in human populations is through observations made upon epidemics in experimental animal colonies Notable among the many contributions are those made upon mouse encephalitis by Theiler (1941) upon lymphocytic choriomeningitis in a mouse stock by Traub (1939) upon infectious ectromelia in mice (mousepox) by Fenner (1948) In each of these studies the specific host parasite relationships were carefully investigated and the mechanism of propagation of the causative agent was elucidated The results were valuable in suggesting what may occur in human experience For example Traub found that in a colony of mice infection with the virus of lymphocytic choriomeningitis occurred regularly and invariably in utero The mice then carried infection throughout their lives Infection of stocks of mice latently infected with this virus may sometimes be revealed by the jolt of an intracerebral injection of sterile broth The adaptation of the virus to Traub s mouse colony finally became so perfect that the mice were all immune and virus could be demonstrated only by the injection of tissue into susceptible mice of another stock

A somewhat different approach by experimental epidemiology is illustrated by the studies of Webster and his associates (1932 1946) on salmonella pasteurella pneumococcus and Friedlander bacillus infections and by Topley and his associates (Greenwood et al 1936 Topley 1942) on salmonella pasteurella and ectromelia virus infections These studies are too extensive to permit detailed review perhaps it will be useful to comment briefly upon the methods used and the knowledge gained

The general procedure was to assemble uninfected animals in unit cages whose arrangement could be altered so as to simulate a community of any desired size A constant regimen of cleaning and feeding was established and appropriate measures taken to prevent the introduction of extraneous pathogenic micro organisms An epidemic was started by introducing into an uninfected animal colony a certain number of animals infected with the microparasite selected for the experiment The course of the subsequent epidemic was indicated by the occurrence of specific deaths proved by autopsy and culture Effort was made to hold all the important factors constant except the one under examination and to note the effect this variable had upon the course of an artificially produced epidemic

It became evident very early in this work that a constant genetic stock of experimental animals was fundamental to control of the host variable As had long been known to the plant pathologists it was found possible within certain limits by selective mating to breed out lines which were relatively resistant or relatively susceptible to infection with a particular micro organism It was demonstrated for example that there may be selected promptly from a hybrid stock of mice of which 40 or 50 per cent die lines in which as high as 95 per cent and as low as 15 per cent succumb following a standard dose of *B enteritidis* This afforded experimental support for the concept of innate differences in resistance to a particular microparasite genetically transmitted in human families lines of descent or races a phenomenon well illustrated in human experience for example by the differences in the host reaction of the white and the Negro races to infection with *Mycobacterium tuberculosis*

The possible importance of nutrition of the host to natural resistance to infection was appreciated If a diet were so poor in quality or quantity as to bring about a state of debility experimental animals whose lives were already jeopardized from the consequences of produced deficiency would have a higher death rate than well nourished animals if subjected to the added insult of infection Obviously it was desirable to hold this factor constant by

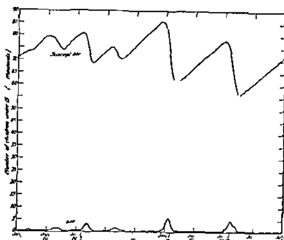


FIG 116 Estimated cases of measles and susceptibles in the Baltimore population under 15 years of age

demic measles if the accumulation of susceptibles were really the prime factor to compare the deduced results with the observed facts and then modify the primary hypothesis Soper s work in turn stimulated W H Frost (unpublished) Lowell J Reed (unpublished) A W Hedrich (1933) A G McKendrick (1940) and E B Wilson and M H Burke (1943) to elaborate the statistical approach to epidemic theory This has elucidated quantitatively the relationships of the principal factors involved and contributed to a rational explanation of the epidemiologic behavior of measles

The fundamental facts with which we start are simple The biologic attributes of the measles virus and the requirements for infective transmission from case to susceptible remain relatively constant *The dynamics of the* mass reaction are due to the flow of the virus through the human population Each new case is due to effective contact with a preceding case in the infectious stage Susceptibles effectively exposed to cases become cases in the next time period cases recovering from the infection accumulate as immunes The susceptibles are being recruited constantly through births and immigrants and depleted through becoming cases and immunes or through deaths and emigration

Upon the basis of a series of logical and reasonable approximations and assumptions Hedrich (1933) made monthly estimates of the child population susceptible to measles in Baltimore from 1900 to 1931 As shown in Figure 116 during a 9 year portion of this period the calculated proportion of susceptibles in the population under age 15 did not rise above 53 per cent or fall below 32 per cent The percentage figures are only approximations but the implications are significant When the proportion of susceptibles was low the incidence of measles tended to be low consequently susceptibles accumulated When the proportion of accumulated susceptibles approached what McKendrick calls a threshold density,' the situation was favorable for the support of an accelerated incidence of cases, or an epidemic During a short period of time the proportion of susceptibles fell rapidly as they became cases and subsequently immunes As the proportion of immunes increased more and more cases failed by chance to make effective contact with susceptibles and the incidence of new cases fell accordingly

It is apparent therefore that the principal factor determining the occurrence of progressive epidemics of measles is the proportion of susceptibles in the population at risk and that the termination of the epidemic wave is due to the dampening effect of the cumulation of immunes and not necessarily to the exhaustion of susceptibles since many escape effective exposure The proportion of susceptibles required to support an epidemic and per contra the post epidemic proportion remaining will vary in every community and even in the same community at different times of the year according to the contact rate " It is much easier to start an epidemic spread of the disease during the winter than in the summer due to changes in the "contact rate which are only partially understood

By utilizing the simplified premises in measles and representing the four principal factors by appropriate symbols it is possible to derive a dynamic equation by which given (1) the number of cases (2) the number of susceptibles (3) the number of the total population and (4) assuming an arbitrary value for the contact rates in one time period of 14 days the number of new cases which will arise in the successive time periods of the same length can be calculated Departures from the course of epidemics predicted from the equation can be explained as due to the

the principal factors which determine incidence and distribution are generally recognized With many if not all however there is need for epidemiologic studies which will define these factors more exactly and establish their relative (crudely quantitative) importance To put the thought in more general terms it is necessary to effectiveness that measures of prevention be directed against those conditions which are of actual importance in the particular situation rather than against the much wider range of conditions which may possibly contribute to the prevalence of the disease Innumerable instances could be cited in which public health campaigns or measures thought to be theoretically sound and rationally conceived failed to accomplish the reduction which was expected

A classic example of critical evaluation of measures to prevent the intrafamilial spread of common contagious diseases is afforded in the development and the use of the secondary attack rate with particular reference to scarlet fever and diphtheria by Dr Charles W Chapin for many years health officer of Providence R I It is related in some detail by W H Frost (1938) in a discussion of the familial aggregation of infectious diseases

The principles and the applications of the method have the merit of yielding information which is easily understood and directly related to the practical problems of the health officer The ultimate epidemiologic unit in a civil community is the family or household a group of people mostly of close kinship sharing a common environment living in close contact in a manner easily described and usually under the eye of a single medical or lay observer The degree of contagiousness of different diseases can be measured by a statistical index derived from familial experience The first case to occur in a family is designated as a primary case A census is made of the exposed members of the family classified by age sex or *other conditions which it is desired to take* into account especially with regard to their past history of having had the specific disease in question or specific immunization against it Then a record is kept of cases occurring in any member of the household *within time limits defined specifically for each disease* with reference to the onset of the primary case so as to include those probably *infected by intra familial contact* It is then possible to summarize the observations on a large number of families and obtain an index of average experience based upon the ratio between secondary cases and exposed persons or exposed persons classified as to age sex relationship previous history immunity status or other quality Schematically represented

Secondary attack rate =

$$\frac{\text{Number of secondary cases}}{\text{Number of exposed persons}} \times 100$$

Buck (1956) illustrates the intrafamilial spread of upper respiratory infections

It is to be noted however that this index is based upon the frequency of secondary clinical cases following the occurrence of a primary clinical case It does not take into consideration the spread by subclinical infections It is useful nonetheless in answering certain questions for example (1) given a case of a communicable disease in the family what is the risk of clinical attack borne by others in the same household within specified periods of time? (2) to what extent can risk of clinical attack be reduced by preventive measures such as removal of the primary case to the hospital immunization of exposed susceptibles? etc It is pertinent to remark in this connection that a practical objective of preventive medicine is to decrease the risk of the penalty of disease and death rather than of a subclinical immunizing infection

While the secondary attack rate is a satisfactory device for evaluation of measures designed to reduce intrafamilial spread it is only indirectly and by inference an indication of their effectiveness in reducing community spread It is obvious that in dealing with a disease such as diphtheria it might be possible to demonstrate that by prompt isolation of the primary case the secondary familial attack rate could be measurably lowered Yet if *there are many individuals who have unrecognized* subclinical infections for each individual who has a clinically recognized attack and both categories are involved in maintaining passage from person to person the effect of prompt isolation of cases upon the incidence in the community as a whole may be so small as not to be measurable

The evaluation of a preventive measure in

providing a uniform and well balanced diet in the test and control groups However it was noted that a diet which was well balanced for normal growth and development was not necessarily well balanced in its effect upon host resistance to infection with a specific micro organism This question has been explored extensively by many investigators in relation to various infections experimentally produced in animals

It has been demonstrated that natural resistance can be influenced by nutrition when the host stock is genetically heterogeneous and the pathogenic population to which it is exposed is heterologous in the sense that it contains an array of variation in terms of capacity to produce disease (Schneider 1951) These studies have advanced the understanding of the underlying mechanisms implied in the term 'resistance to susceptibility' but diet was of very limited importance as one of the variables affecting results of experimental epidemics produced by *Salmonella enteritidis*

The variability in the biologic potentialities of the strains of infecting micro organisms employed received considerable attention A theory had been advanced that the rise of a progressive epidemic is due principally if not wholly to a progressive increase in virulence of the specific agent with the rapid human passage As the infectious agent encounters more resistant individuals there is a progressive decrease in virulence resulting in less frequent passage and a falling off of the number of new cases in successive time periods To test this theory methods were devised by Webster for measuring the 'virulence of a specific strain of micro organism for groups of mice by administering a fixed dosage In artificially produced epidemics sample cultures were obtained from animals dying at various stages Comparative titrations were made on strains from epidemics of pasteurellosis in rabbits chickens and mice Similar titrations of two serologic types were made during the course of mouse typhoid infections in mouse populations A total of 300 or 400 titrations were made under many conditions to test the theory of fluctuating virulence The results were invariably negative and showed a constancy and fixity of disease producing power of a given strain of organisms under all conditions of natural infection

From his experience with experimental epidemiology, Webster was inclined to believe that in all instances changes in biologic potentialities of specific microparasitic species are of little or no importance in determining the secular rise and fall of epidemic waves While this may be true for many parasitic species, there are some which are more unstable and have a greater capacity for selective variation in pathogenicity (Burnet, 1951) The development of sulfadiazine resistant or penicillin resistant strains of bacteria is a pertinent indication of what may happen in nature

In a series of experiments it was demonstrated that when infected animals were introduced in a closed universe of susceptible animals the ensuing epidemic quickly subsided as susceptibles died or became immune although a few escaped infection An epidemic started in this manner could be maintained in an open universe if sufficient susceptible recruits were added at regular intervals The course of the epidemic was modified by the relative number of susceptibles and the rate at which they were added If the conditions were held relatively constant the balance between the microparasitic and the host population tended to reach a stabilized equilibrium This was violently disturbed by a major change in the contact rate which was accomplished by bringing a large number of animals previously dispersed in small single cages into a single colony in a large cage

These and other experiments added support to some of the generalizations derived from experiences with epidemics in human populations under natural conditions They emphasized particularly the accelerating effect upon incidence of an inflow of susceptibles into an infected community and of aggregation of individuals into large groups (crowding) and per contra the dampening effect upon incidence of accumulation of immunes *But the actual quantitative importance of each of these factors varies with the disease its mode of transmission the host relationships involved and the local circumstances*

EVALUATION OF PREVENTIVE MEASURES

With most of the common contagious diseases knowledge has advanced to a point where

afforded The *criteria used in clinical diagnosis* must be clearly stated The resulting differences in attack rates in the two groups must be sufficiently large to be statistically significant

This is a basic outline of the general approach to such problems There are always many perplexing circumstances and occurrences tending to disturb the results for which allowance must be made in some manner Illustrations of this type of epidemiologic studies designed to evaluate critically an immunization procedure will be found in Bell (1941) on pertussis prophylaxis with 2 doses of alum precipitated vaccine and Francis on poliomyelitis vaccine (Salk) (1957) An example of a study planned to assess critically the value of prophylactic administration of a drug will be found in the report on the dynamics *of meningococcal infections and the* effect of chemotherapy by Phair and Schoenbach (1944)

EPIDEMIOLOGIC FEATURES OF NONCONTAGIOUS DISEASES

A considerable proportion of the diseases of man are due to microparasites which have multiple host relationships in nature Man plays the role of a more or less innocent by*stander* who *suffers accidentally from the* struggle which is going on among other forms of life which are part of the world in which he lives or which he invades in competition for food and shelter Many of his diseases are the result of association with animals and birds which he has brought into his domestic economy others are the result of contact with wild animals and still others are due to the fact that he is occasionally an available source of food (blood meal) for various species of arthropods The pathology of the infection in man is of such character that although the microparasites may undergo considerable multiplication in his tissues they either fail to make an effective exit in the secretions or the excretions or do so in such small numbers and in such a short period of time that they cannot maintain continuous man to man passage or they fail to make effective exit from the peripheral circulation except through the mediation of an arthropod vector To understand and interfere effectively with transmission to man requires knowledge of the underlying *infection chains* These have been reviewed in complete outline by K F Meyer (1948) in *The animal kingdom A reservoir of human disease*

In the accompanying table (Table 60) there is a summary of the principal host relationships of bacteria and fungi transmitted to man by contact with animals or birds or by arthropods They have been divided into two groups (1) those that are dependent upon domestic ecology and (2) those that are dependent upon the ecology of wild life Under appropriate chapter headings what is known about the natural propagation of each of these species of microparasites has been set forth in some detail However some generalizations may be drawn with regard to the epidemiologic pattern of the two groups The first group is associated with practices in animal husbandry The occurrence of the disease in man is an indication of its existence and chain transmission in the herds the flocks and the domestic animals which he tends He may be infected by direct contact with the secretions *the excretions and the tissues of living animals* and birds or by their products after slaughter Transmission may be rather immediate to those handling livestock and their products rather remote to consumers of contaminated *food or milk or still more distant to those* working with hides wool hair etc Except as transmitted by contaminated food and milk these diseases show an occupational selection for farmers veterinarians dairymen workers in abattoirs workers in factories handling hides wool hair etc They are sporadic in their human occurrence and are limited in their geographic distribution to localities or foci where the practices of animal husbandry are of such a character as to support propagation of the infectious agent

Upon the medical epidemiologist devolves the necessity of tracing sources of human infection of ascertaining the conditions under which transmission occurred and of instituting necessary precautions so far as practicable to obviate exposure In this activity he collaborates with the veterinary epidemiologist who is also concerned with reducing economic losses to animal husbandry More exact knowledge of how these diseases are being maintained by infection chains in herds and flocks is accumulating constantly Measures to di

reducing the incidence of a disease in a large population unit such as a city may be illustrated by the studies which have been made of the effect of changing from a contaminated to a safe water supply There have been two methods of approach In one the incidence of typhoid fever for several preceding years in the same community has been compared with the incidence of this disease in the years immediately following the change that is comparing the attack rate expected on the basis of past experience with the observed attack rate This is valid provided that it can be shown that there is an abrupt decrease coincident with change that the completeness and the accuracy of the reporting of cases and deaths is of the same order over the whole period that no other factor or factors could reasonably account for the sudden difference The second method and one that can seldom be realized now is by concurrent comparison of two populations alike in all pertinent respects and differing only with regard to their water supply A classic example of the latter method is presented in the observations of John Snow (1865) during the epidemic of 1854 55 comparing the incidence of cholera among the consumers of water supplied in certain districts of south London by two competing companies The experiment too was on the grandest scale No fewer than three hundred thousand people of both sexes of every age and occupation and of every rank and station from gentlefolks down to the very poor were divided into two groups without their choice and in most cases without their knowledge one group being supplied with water containing the sewage of London and amongst it whatever might have come from the cholera patients the other group having water quite free from such impurity

Nature however seldom sets the stage for a scientific experiment in such manner that it is possible to observe two population groups alike in all important respects except with regard to one factor So it becomes necessary to set up such groups artificially if our many questions as to the effectiveness of control measures are to be answered Unusual opportunities for such studies were afforded in military organizations during the war The many considerations which must enter into investigations of this type are illustrated by a study of the effect of double bunking in barracks and of oiled floors and bedding on the incidence of respiratory disease in new recruits (Commission on Acute Respiratory Diseases and Commission on Air Borne Infections, 1946)

EVALUATION OF IMMUNIZATION AND CHEMOPROPHYLAXIS

The same kind of considerations enter into epidemiologic investigations designed to evaluate the prevention of a specific infectious disease by an immunization procedure or by the prophylactic administration of antibiotics or chemical compounds The preliminary work in testing effectiveness and safety are carried out in the laboratory upon experimental animals When sufficient evidence has been accumulated to justify the use the final evaluation of the efficacy of such agents can be obtained only by human trial Furthermore these observations must be so controlled as to merit scientific acceptance of results Failure to meet this necessity has led in many instances in the past to the exploitation of biologic products and chemical substances which was unwarranted and at times actually detrimental It has become painfully evident that evaluation by clinical impressions is unreliable

The basic requirements of critical trials upon human beings are well known but the actual conduct of such an experiment is fraught with practical difficulties Ideally two groups of persons (a test and a control group) are placed under observation They must be alike in all essential respects particularly those which relate to their susceptibility at the beginning of the experiment and their exposure to natural infection throughout the period of observation The substance to be tested must be administered without discrimination if possible alternate individuals receiving a placebo or a blank It is highly desirable that neither the subjects themselves nor the investigator who is responsible for their subsequent follow up and observation should know who has received the test material and who has not In this manner errors due to unconscious human bias may be obviated Individuals of both groups must be examined with equal frequency care for equal periods and for a sufficient length of time to ensure an adequate test of the protection

of the biting wound as with the flea X cheopis in the transmission of plague bacilli The agent may make an effective entrance into the body of the arthropod host multiply in the intestinal tube and penetrate the walls of the gut to invade other tissues as with the louse (P humanus) in man to man transmission of B recurrentis in relapsing fever In the hemocele of the louse the spirochetes are in a blind alley They cannot be transmitted by the bite or the feces of the insect When the louse is crushed the skin or the mucous membrane of the human host is contaminated by the spirochete laden blood of the louse and the organism gains entrance into the body through the skin scarified by scratching or through the intact mucous membrane of the conjunctiva In the tick (O moubata) these spirochetes (B recurrentis) penetrate the stomach wall and enter the hemocele From there they invade various tissues including the salivary glands the coxal glands and the ovaries Thus they make an effective exit as transmission to the vertebrate host is effected by the injection of salivary secretions during feeding or by the coxal fluid which is secreted by some ticks while feeding or by contaminated feces In the ovaries the spirochetes penetrate the eggs hereditary transmission may continue for several generations without reinfection by feeding upon infected blood The tick thus acts as a reservoir as well as a vector Ticks (*H leporis palustris D andersoni* and *D variabilis*) play a similar role in tularemia After feeding on an infected rabbit the tick harbors *Bacterium tularensis* in its coelomic fluid rectal sac and feces In its arthropod host *Bacterium tularense* survives the winter and may be transmitted from stage to stage through the egg The tick plays the part of a biologic vector in propagating the disease in the rabbit and at the same time contributes to the maintenance of a reservoir from which other wild animals and birds are infected Thus the arthropod host as well as the vertebrate host has various degrees of responsibility in the propagation

The highly specialized ecology required for the maintenance of these three diseases can exist only in limited areas Therefore geographic distribution is localized or focal The seasonal distribution of human cases is determined by the seasonal activity of the vector species or the predatory habits of man Tularemia is an endemic disease So long as plague is limited to wild rodents (sylvatic plague) human cases are few and far between When it spreads to commensal rat populations which have a high X cheopis index it occurs in devastating epidemics Relapsing fever due to tick bite is sporadic When it becomes propagated by the louse man louse chain it occurs in epidemics

One of the functions of epidemiology is to assemble the observations made upon the various phases of the natural occurrence of the specific microparasite in relation to its vertebrate and arthropod hosts and by inductive reasoning arrive at a concept that would explain the principal and subsidiary transmission chains Then this concept is tested by further observations and experiments leading to modifications as new knowledge develops in the usual manner of scientific advance How such investigations may proceed is illustrated by the recent identification of the mite as a vector of rickettsialpox which made its appearance in the Queens area of New York City in the summer of 1946 Rapid epidemiologic investigation indicated that inasmuch as patients suffering from the Queens disease were all living in one group of houses about 3 blocks square while persons in other houses in the neighborhood were unaffected it was distinctly sporadic and focal in distribution The presence of mice in the houses where the patients lived led to the hypothesis that a mouse ectoparasite might be the source of the disease Recovery and identification of the etiologic organism by injecting the blood of patients into laboratory animals had just begun and the long process of catching mice and combing them for ectoparasites was in process when an insect exterminator helped solve the problem He was successful in collecting some mites from the crevices of cellar walls in the neighborhood and presented them to the scientific investigators The causative microparasite was quickly recovered from these mites then from the patients and later from the mice The fortuitous collection of the exterminator saved months of investigation

In conclusion the following quotation from Frost (Snow 1936) is appropriate Epidemiology at any given time is something more than the total of its established facts It in

TABLE 60 PRINCIPAL HOSTS OF BACTERIA AND FUNGI TRANSMITTED TO MAN BY CONTACT WITH ANIMALS AND BIRDS AND BY ARTHROPODS

	Animals										Arthropods			
	Horses	Cattle	Swine	Sheep	Goats	Dogs	Cats	Commensal Rats	Wild Rodents	Domestic Fowl	Flies	Fleas	Ticks	Lice
Domestic														
Actinomycosis (*Actinomyces bovis*)		+												
Anthrax (*Bacillus anthracis*)	+	+		+	+									
Brucellosis (*Brucella*)	+	+	+	+	+									
Haverhill fever (*Streptobacillus moniliformis*)								+						
Leptospirosis (*Leptospira*)						+		+						
Listerellosis (*Listeria monocytogenes*)		+	+	+	+				+	+				
Rat bite fever (*Spirochaeta morsus muris*)								+						
Ringworm (*Dermatophytes*)	+	+				+	+	+						
Salmonellosis (*Salmonella*)		+	+	+		+	+	+		+				
Swine erysipelas (*Erysipelothrix rhusiopathiae*)			+	+						+				
Tetanus (*Clostridium tetani*)	+	+												
Tuberculosis bovine (*Mycobacterium tuberculosis*)		+												
Wild														
Plague (*Pasteurella pestis*)								+	+			+		
Relapsing fever (*Borrelia*)									+				+	+
Tularemia (*Bacterium tularense*)				+				+	+		+	+	+	

minish the opportunities for propagation by contact or to increase resistance by artificial immunization are being developed and evaluated

The group of diseases associated with the *ecology of wild life presents more complex* problems The principal chain of transmission is maintained by alternation of vertebrate and arthropod hosts The relationships of the specific microparasite to both must be elucidated The lead to the arthropod vector or vectors usually is supplied by epidemiologic observations These may be of a very crude character no more than popular ideas associating the occurrence of a disease with certain insect bites The people of certain valleys in Japan *suspected that tsutsugamushi* disease was conveyed by the mite many years before the role of this arthropod in transmission was established scientifically Entomologic investigations are based upon such leads The approach is usually along two lines—to transmit the infectious agent in human or animal experiments or to recover the etiologic agent from suspected arthropods captured in nature Distinction is made between arthropods which are experimentally infectable and those which are found to be naturally infected Of those found to be naturally infected, study establishes *whether or not they are efficient* vectors The efficiency of a vector depends upon many factors including its numbers, distribution habits and susceptibility to infection with the agent

Arthropods vary in reaction to microparasites as do vertebrates The agent may not make an effective entrance into the body of the arthropod host and accordingly be unable to multiply The infectious material is carried from the infected to the noninfected vertebrate mechanically Thus the deer fly *C discalis* is a mechanical vector of tularemia The infectious agent may be successful in making entrance into the body of the arthropod host with a blood meal remain *limited to the intes*tinal tube undergo multiplication and make an effective exit either through regurgitation at the time of biting or by fecal contamination

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Principles and Practice of Diagnostic Medical Bacteriology

INTRODUCTION

The most exact diagnosis is furnished by the isolation and the identification of the recognized causative agent Ideally this requires the application of all available knowledge but the usual pressure of work in the laboratory limits what can be undertaken in a routine service So it becomes essential to develop a scheme of procedure designed not to miss any well known pathogenic micro organism and capable of being expanded to meet special circumstances and requirements

The isolation and identification procedure is not always possible since certain organisms cannot yet be grown or only with great difficulty and many another requires special materials and conditions It is also impossible when the lesions are not accessible and when organisms are scarcely obtainable at particular stages of the disease Therefore the scheme of procedure must include means of recognizing antibodies formed by the patient during the course of infection or free antigens liberated into the body fluids and tissues by the invading micro organisms This too has its limitations Either the known applications of immunity reactions are not delicate enough or the reacting substances are present in too minute quantity for detection Above all no laboratory can possibly maintain every requisite antigen or antibody necessary for this purpose The upshot of the situation is that no laboratory can be expected to be ready to perform every conceivable test or examination at short notice and without warning

A well appointed bacteriologic laboratory has greater functions than mere routine diagnostic procedures It has long been evident that selective and specific therapy should be guided by the laboratory findings and directed in consultation with the bacteriologist At the present time with selective antibiotic agents and sulfonamide drugs and with the diverse possibilities of the development of resistant strains in the course of treatment the importance of the laboratory is greatly increased It has become necessary to determine the susceptibility of strains to the various available therapeutic substances and select the most effective single drug or suggest the most promising combination This places the burden of responsibility for the choice of treatment and frequently also for the dosage upon the bacteriologist a function which can be performed satisfactorily only if all pertinent information about the patient is made available The wide spread use of antibiotics and other chemotherapeutic agents is leading to changes of the commensal flora to superinfections and to the evolvement of new disease entities Contrary to earlier expectations these drugs have created new problems in the management of infectious diseases and the contributions of

cludes their orderly arrangement into chains of inference which extend more or less beyond the bounds of direct observation Such of these chains as are well and truly laid guide investigation to the facts of the future those that are ill made fetter progress'

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specimen containers This receives all too little attention as a rule and can be serious At times the entire specimen can be lost in a cotton plug (ureteral urine) or if there is delay in delivery it may be contaminated through a wetted plug In any case it is deplorable technic and indicates a lack of instruction and supervision by those in responsible positions The danger of spread of infection by imperfectly trained help is frequently overlooked and unwarranted reliance on them commonly relates to quite important procedures despite legal restrictions on less important matters This applies most frequently to the collection of specimens from male patients

Wherever possible specimens should be taken before any treatment with sulfonamides or antibiotics is started or before wounds are treated with antiseptics If this is unavoidable the specimen label should tell what it may contain in order that suitable precautions be taken by the laboratory This is becoming increasingly essential

One other contributing factor to be considered is the stage of the disease The clinician should take into account which type of specimen is most likely to yield results during a particular phase of disease and realize that when this selection is not made negative findings do not exclude the correctness of the diagnosis a good example is afforded by stool blood and urine in typhoid fever or leptospirosis It is also a common mistake to submit samples of serum for agglutination reactions at a time when antibody response cannot be expected much time and energy can be expended looking for Leptospira in the blood at an entirely unsuitable stage of the disease

EXAMINATIONS OF MATERIAL FROM PATIENTS

One or several of the following methods are employed depending on the material received and the suspected type of infection microscopic studies on stained or unstained material including darkfield cultivation isolation in pure culture and identification of the microbes by all available means and technics inoculation of suitable animals serologic studies of the patient s serum and serologic typing of isolated strains testing of the isolated micro organisms for their sensitivity to antimicrobial agents

The usefulness of the diagnostic laboratory is determined by the speed with which the results of all investigations become available to the clinician Preliminary reports provided that they are based on experience and sound judgment may aid in the management of the patient

Blood Cultures

The exact diagnosis of many systemic infections is accomplished best and most speedily by the isolation of the causative organism directly from the blood Bacteria may enter the blood stream by one of the following routes (1) from local sites by spread through the lymphatics or infected thrombi and emboli and (2) by direct introduction of bacteria into the blood either by biting insects or through accidental introduction of contaminated materials into the circulation The first mentioned pathway is by far the more common and quite frequently the invasion of the bloodstream by bacteria is the first clinical manifestation of some local lesion The type of organism isolated from the bloodstream may provide important information on the possible site of the primary focus When the classic findings of a septicemia—chills high fever prostration—are present blood cultures are requested immediately and in such instances the isolation and the identification of the causative organism rarely encounters difficulties The situation is more complex when dealing with transitory bacteremias in which the micro-organisms are present in the blood during limited periods only or when the infecting organism is of a type which causes low grade subacute or chronic infections and is present only in small numbers A still more difficult task arises for the laboratory if the generalized infection is due to a rare causal agent or one that requires highly special methods for isolation

In each of these cited examples different considerations are involved for the laboratory In the acute septicemia speed in the complete identification and testing for sensitivity of the isolated strain is the most important aspect *Staphylococcus pyogenes* and *Streptococcus pyogenes* are the most common organisms found but other bacteria may give rise to

the diagnostic laboratory are equally or even more important after the diagnosis is made than before

In addition to these basic functions the laboratory in many instances has taken on new duties such as advice and guidance in the establishment of bone and artery banks and supervision of the proper maintenance and safety of such services

In recent years diagnostic laboratories in most regions have been concerned with investigations and possible means of prevention of hospital cross infections mainly due to staphylococci but also to other organisms To secure the effective performance of these manyfold functions and a progressive increase in the knowledge of infections and their management, it is essential to promote the closest possible *co operation between the laboratory and the* ward Consultation as between equals should be the rule and not the exception There is as much necessity for the bacteriologist to have a good knowledge of the clinical problems and difficulties as for the clinician to appreciate the possibilities and the limitations of the laboratory with the purpose of benefiting the patient and of advancing knowledge by the co ordination of laboratory findings and clinical observation

Co operation between laboratory and ward is not confined to senior members of the staff It involves those concerned with the collection of specimens and their delivery to the laboratory and no less those who supply the information upon which the laboratory worker judges what procedure and technic will give the most useful results It involves the supply by the laboratory of adequate equipment for the collection of various suitable specimens and a statement of the information required and a willing guidance in special cases Thus *it evidently depends upon the heads of* laboratories and clinical services to promote co operation and to organize procedure in the light of mutual understanding good fellowship and scientific curiosity

GENERAL CONSIDERATIONS ON THE COLLECTION OF SPECIMENS

The first step in the isolation and the identification of pathogenic bacteria is the collection of specimens to be examined This fundamental step often does not get the attention to detail it requires, even though the results and the usefulness of bacteriologic studies are dependent as much on the proper time and method of collecting the specimens as on their proper handling in the laboratory The laboratory report can state only what is found in the specimen submitted Failure to isolate the causative organisms of an infectious process is often due to improperly taken specimens or to lack of information on the clinical condition for guidance in the selection of suitable media and technic, or of other methods necessary for arriving at a diagnosis Too often in a busy ward the taking of specimens is left to persons who have no proper understanding of the requirements and no knowledge of the responsibility and the consequences

Material should be collected with proper *precaution from active lesions or situations* where the suspected organisms are most likely to be found It is often necessary to select particular portions of available material (such as dysentery stools, sputum, actinomycotic pus etc) and whenever possible such selection should be left to the laboratory At times the patient must contribute in the collection of a specimen and then should be fully instructed and encouraged by being given a reason Specimens should be amply sufficient to allow of every necessary examination particularly if several are asked for and placed in sterile containers to avoid misleading or hampering contaminations Once collected the specimen should reach the laboratory as soon as possible to ensure its being in a good state and this is of particular importance when the organisms concerned are liable to injury by drying by exposure to air and by prolonged chilling Sometimes specimens are unavoidably small in amount and these should not only be transmitted to the laboratory quickly *but also should be collected* in special containers At times it is necessary to take the media to the bedside or the patient to the laboratory (Neisseria Treponema etc) and some specimens should be collected by the bacteriologist For organisms requiring unusual conditions arrangement should be made beforehand with the laboratory

An important source of danger to the laboratory worker to the porter carrying the specimen to the laboratory to nurses or to other patients is the soiling of the outside of

cases of brucello is met with in hospital or private practice because even repeated negative cultures do not exclude the diagnosis A great many methods and media for the isolation of brucella from the blood have been described but so far no uniformly reliable method is available (Foston 1941 Huddleson 1943 Wise 1943) The number of successful isolations from presumptive cases of acute brucellosis might well represent a criterion of the standard of individual laboratories

Rat bite fever relapsing fever and leptospirosis are a challenge for the bacteriologist Periods of pyrexia may be the outstanding feature in all these diseases and the differential diagnosis based on clinical observation without laboratory aid is often impossible Their successful diagnosis requires ingenuity and close co-operation between the physician and the experienced bacteriologist

Rat bite fever may be caused by two different micro-organisms *Streptobacillus moniliformis* and *Spirillum minus* (For a different opinion see Dolman et al 1951) If there is a history of a bite by rats or other rodents the laboratory will mainly have to differentiate between the two etiologic agents If there is no evidence of animal bites a difficult diagnostic problem may arise as the clinical manifestations of septic temperatures frequently associated with erythematous or petechial skin rashes and joint pains may be due to a variety of unrelated agents *Streptobacillus moniliformis* can be grown on enriched media (20 to 30% serum or ascitic fluid) but so far the cultivation of *Spirillum minus* has not been successful Inoculation of mice and guinea pigs with the blood is the most promising diagnostic procedure with due precaution that the animals be initially free of naturally acquired Spirillum infection Then the Spirillum can be demonstrated by darkfield and simple staining in the blood of the susceptible animals

Leptospirosis is world wide in distribution and was thought to occur mainly in persons having contact with water polluted by rats and water frequenting rodents but in recent years the widespread distribution of leptospira in our environment and in domestic and wild animals has been understood Many disease processes in man caused by these organisms have been recognized and diagnosed (Kalz 1957) In the typical form of Weil s disease with jaundice the laboratory demonstration of *Leptospira icterohaemorrhagiae* may be confirmatory of the clinical diagnosis but in a large number of cases jaundice is lacking and the diagnosis is dependent exclusively on the laboratory findings Leptospira can be cultivated relatively easily from infected animals and sometimes directly from patients They have also been demonstrated although very rarely by darkfield or Giemsa stained films in the patient s blood during the first 4 days of the disease After the eighth day cultivation or examination of the blood is usually unsuccessful and should be replaced by examination of the urine Intraperitoneal and subcutaneous inoculations of blood or urine into guinea pigs or golden hamsters is a reliable diagnostic method although the susceptibility of these animals may vary considerably for different Leptospira strains

Relapsing fever is caused by various species of Borrelia and according to the species is transmitted by lice or ticks The diagnosis depends on the demonstration of the organisms in the blood either directly by darkfield examination and in stained films during the rise of fever or indirectly by inoculation of suitable animals (young white rats) Although cultures have been claimed on enriched media growth is not reliable However survival of spirochet s in drawn blood even in culture media is demonstrable for some 30 days and longer in defibrinated sheep blood Th clinical picture of the acute initial phase resembles so greatly other diseases that differential diagnosis without laboratory aid is impossible

Blood culture may be very helpful in the preanemic period of Oroya fever and should be done in all patients coming from the mountain regions of Peru Ecuador or Colombia and showing suggestive signs and symptoms fever joint and bone pains The identification of *Bartonella bacilliformis* should cause no difficulty if the patients history is considered

The technics of blood cultures vary somewhat in different laboratories however the main point is to preserve absolute sterility throughout all manipulations Contaminated cultures may lead to difficulties of interpretations or worse to erroneous and misleading reports The amount of blood withdrawn should be sufficient to allow for inoculation of all necessary media possible animal inocu

this acute clinical picture anaerobic strepto cocci coliforms *Bacteroides Cl perfringens* and more rarely others

A different problem both from the clinical and the bacteriologic aspect is presented by transitory bacteremia or septicemia which occur at certain stages of well defined disease entities In a fairly high percentage of cases (26% of pneumococcal pneumonia) blood stream invasion precedes or accompanies the early stages of pulmonary lesions and then may be of prognostic significance The sys temic infection indicates greater severity of the disease process, and the treatment sched ule should be chosen accordingly Most in vestigators agree that a bacteremia precedes every case of meningococcal meningitis and that the latter represents a metastatic lesion of the systemic infection The blood stream invasion may be transitory and cause only minor clinical disturbances and only the lo calization of the metastatic lesion leads to overt disease On the other hand the invasion of the blood stream by meningococci may lead to a fulminant or a chronic septicemia with or without metastatic involvement of the meninges Blood cultures are then particularly important because differential diagnosis rests entirely upon the demonstration of the organ ism The widespread petechial lesions which are commonly found in meningococcal sep ticemia are due to bacterial emboli and the bacteria can be isolated from such lesions

Enteric fevers are systemic infections and during their early stages the cultivation of the blood is the only reliable means of diag nosis The early recognition is important not only for treatment of an individual case but also for the prevention of a possible epidemic Transitory bacteremia or septicemia occur dur ing certain stages of other diseases In plague invasion of the blood stream occurs in severe and fulminant cases The isolation of *Pas teurella pestis* presents no particular difficulties in these and may be of prime importance in regions where plague is almost unknown or occurs as an isolated case

A bacteremia of a few days duration oc curs fairly regularly in the early stages of tularemia The isolation of *Pasteurella tula rense* either by culture or by animal inocula tion is especially valuable diagnostically in those cases which show no primary lesions The rather exacting growth requirements of *Pasteurella tularense* must be kept in mind as the 'routine media will not permit its growth Therefore the physician must provide some indication of the possible nature of the infec tion The occupation of the patient a history of arthropod bites or of handling possibl source animals will be sufficient to guide the laboratory in the selection of appropriate media and other suitable diagnostic methods

There is a group of systemic infections of subacute chronic or remittent type in which the diagnosis is entirely dependent on the iso lation of the causative microbe from the blood and this group presents the greatest challenge to the physician and the bacteriologist Com monly in these bacteria are present in small numbers and at irregular intervals and often a great deal of patience and persistence is required before a definite diagnosis can be established These difficulties are aggravated if various ineffective treatments precede bac teriologic examinations Patients suffering from such types of infections are rarely in immediate danger and the advantages of a correct diagnosis even if requiring some time will be of greater benefit than trial and error therapy The most common diseases in this group are subacute bacterial endocarditis and brucellosis Before the discovery of antibiotics subacute bacterial endocarditis was almost in variably fatal but modern treatment can be very rewarding if the diagnosis is made before irreparable damage to the cardiac and circu latory system has taken place A prerequisite for the successful treatment of this disease is the isolation the identification and the testing of antibiotic susceptibility of the microbe re sponsible in the individual cas The great majority of infections is due to one of the species of the viridans group of streptococci rarer cases are caused by one of the entero cocci however many other bacterial species (Shilling 1939) may be involved on occasion

The diagnosis of brucello is is one of the most difficult and in many ways the most frus trating task for the bacteriologist and the physician Even during the acute phase of the infection the isolation of the organism from the blood stream is not regularly successful and during the subacute and the chronic stages it is only rarely accomplished

It is difficult to estimate the number of

sputum for cultivation They are usually a reliable index of the organisms causing the respiratory tract infections The paranasal sinuses are sterile in health or contain at most only a few saprophytes which may originate from contamination during the collection of specimens In disease the flora of the sinuses may be quite varied and contain almost any kind of bacteria Infection of the maxillary antra may occur through apical tooth abscesses and in such cases different types of anaerobic organisms will be found The inspection of the specimen its description as mucoid mucopurulent or frankly purulent its odor and the study of a gram stained film will be a guide in the choice of the media Swabs from throats or tonsils are taken most often in cases of acute tonsillitis and will yield *Streptococcus pyogenes* in the majority of cases The proof of or the exclusion of diphtheria is requested from the laboratory and is very important because of the occasional case of modified diphtheria following an imperfect response to immunization with toxoid These cases show no more than a fairly profuse serosanguinous exudate which is more often nasal than pharyngeal The physician must realize that the complete laboratory diagnosis takes too long to be of immediate diagnostic value and that the decision as to whether or not antitoxin should be used rests with him If a typical membrane is sent to the laboratory and the smear shows characteristic organisms in large numbers this fact should be reported to the clinician immediately A negative smear of a poorly taken throat swab does not exclude the diagnosis of diphtheria The diagnosis of Vincent s angina can be confirmed microscopically and best by darkfield examination of fresh specimens which reveal the characteristic active movement of the organisms and their solid appearance and delicate form The cultivation of the fusiforms is hardly worth the great trouble and *Borrelia vincenti* cannot be grown at all Films stained by gentian violet or gram will show the characteristic picture of spirochetes and fusiforms in very large numbers *Borrelia vincenti* cells are thin with open coils and pointed ends and show very active serpentine movement *Fusobacterium plauti vincenti* is of medium size thin and gradually tapered Both are more delicate than related species The diagnosis is not easy and should not be based on a few spirochetes and fusiforms especially when these are of large size sluggish and doubly contoured as seen by darkfield examination Large round or oval budding yeast cells (gram positive) and occasional short thick filaments will be found in smears of thrush lesions—whitish or creamy easily removable membranes *Candida albicans* is the causative fungus and can be grown and identified

The interpretation of cultural findings from all these specimens requires experience and judgment The normal commensal flora of these regions has to be known not only as to types but also as to relative numbers of the various organisms

It is one of the major tasks of the diagnostic laboratory to look for the unusual The observant bacteriologist may find organisms associated with a particular disease process and establish in this manner new disease entities *Listeria monocytogenes* is a good example of an organism which has been isolated from throat swabs on occasions by workers particularly interested in this microbe and is missed if only routine is carried out *Staphylococcus pyogenes* is present in a fairly large percentage of healthy people and therefore not necessarily related to a disease process yet on occasion its finding in nasal or throat swabs may indicate an incipient infection of the respiratory tract particularly in infants and young children

Sputum Bronchial Secretions and Other Specimens from the Lower Respiratory Tract

The organisms most frequently associated with acute bacterial infections of the respiratory tract are pneumococci influenza bacilli Friedlander s bacilli various streptococci and staphylococci In infants fulminant and rapidly fatal cases of tracheobronchial staphylococcal infections have to be differentiated from tracheal diphtheria and from severe tracheitis due to *Hemophilus influenzae* In typical cases of lobar pneumonia the sputum is rusty and shows practically nothing but pneumococci which can be easily isolated and if desired typed directly by the quellung reaction Acute or chronic infections due to *Klebsiella pneumoniae* (Friedlander s bacillus) show a thick tenacious sputum There

lation and any other required test Arterial puncture appears to be an unnecessary complication The cultivation of bone marrow may present advantages over blood cultures in certain instances (Brucella Histoplasma) The media used depend upon the suspected micro organisms and partly on local preferences The interpretation and the diagnostic evaluation of positive findings from blood cultures require no further elaboration apart from the *already mentioned problem of contaminations*

Cerebrospinal Fluid

In any suspected case of meningitis the study of spinal fluid constitutes an essential part of diagnosis Spinal fluid must be collected with sterile precautions and sent to the laboratory as soon as possible A fairly wide variety of micro organisms may be responsible for infections of the central nervous system or more specifically the meninges The clinical picture and course—acute subacute or chronic—allow for certain probabilities of the species of organisms involved The acute purulent forms are caused most commonly by meningococci pneumococci *H influenzae* (particularly in children) less commonly by staphylococci streptococci and organisms of the colon typhoid group and occasionally by other bacteria In recent years attention has been focused on *Listeria monocytogenes* as causal agent of acute meningitis or meningo encephalitis Although we are almost certainly not dealing with a new type of infection little attention has been given to this organism as an etiologic agent and for this reason it probably has been frequently overlooked This view is supported by the relative frequency with which the diagnosis is made in laboratories *having special knowledge and a particular* interest in this type of infection In the newborn infant the meconium is a most valuable specimen to examine (Seeliger 1955) Subacute or chronic cases of meningitis may be caused by a number of micro organisms depending upon the geographic location *Mycobacterium tuberculosis* or various fungus species (*Cryptococcus neoformans* Nocardia Candida) may be found with greater frequency Leptospira are being recognized more frequently as causative agents of so-called "aseptic meningitis"—a disease which often goes undiagnosed because of the special conditions required for the isolation of these organisms Viruses as etiologic agents are outside our framework of discussion

Exudates from Eyes Ears Nose Throat and Paranasal Sinuses

Most of the common pyogenic organisms can cause conjunctivitis *Staphylococcus pyogenes Streptococcus pyogenes Haemophilus influenzae* pneumococcus rarely meningococcus Severe forms are caused by gonococcus and *Corynebacterium diphtheriae* Angular conjunctivitis is usually caused by *Moraxella lacunata*, and conjunctivitis with ulcerations by *Moraxella liquefaciens* both characteristic short gram negative diplobacilli It is necessary to remember that (1) *Moraxella lacunata* will grow only on Loffler s inspissated serum and must be subcultured from the pits it makes in the serum and that (2), although *Moraxella liquefaciens* also grows well on Loffler s serum its great proteolytic activity renders isolation difficult Fortunately *Moraxella liquefaciens* will grow satisfactorily on blood agar

For the demonstration of cytoplasmatic inclusions actual epithelial scrapings have to be sent to the laboratory The inclusion bodies of trachoma and inclusion conjunctivitis can not be differentiated but the clinical findings will solve this problem Laboratory facilities are seldom adequate to perform positive identification of other viral infections of the eye Infections of other structures of the eye—iritis iridocyclitis etc—may cause serious diagnostic problems chiefly because of the difficulty of obtaining suitable specimens Discharges from ears in acute subacute or chronic otitis media usually yield pyogenic organisms and have to be treated accordingly Proteus and pseudomonas are often present in discharges from chronic otitis media as secondary invaders either singly or mixed Very rarely will *Actinomyces israeli* or *Mycobacterium tuberculosis* be found in these specimens but the possibility must be considered particularly in the light of the history of the patient In chronic infections of the ear chiefly the external ear fungi especially Aspergillus but also others may be involved and should be thought of in the choice of media Nasal secretions may yield any of the respiratory pathogens In babies and infants pharyngeal and nasal swabs have to be used instead of

aggravate and disseminate throughout the body infections by fungi of greater pathogenicity which otherwise would remain localized The demonstration and the isolation of fungi from sputum or bronchial excretion is not always accomplished easily The addition of certain antibiotics to special media facilitates the isolation of fungi by suppressing part of the bacterial flora However this technic cannot be used for the isolation of *Actinomyces israeli* Its growth requirements are exacting and it is susceptible to some antibiotics The isolation of this slow growing microbe from the mixed flora of sputum may present a major laboratory problem No indirect methods of diagnosis are available and as *Actinomyces israeli* may occur on the mucous membrane of the mouth of healthy people the isolation from sputum cannot be considered conclusive evidence of the etiologic relationship to the suspected pulmonary lesion The isolation of *Candida albicans* from sputum or bronchial secretions presents no difficulties but creates a problem of interpretation Pulmonary moniliasis and aspergillosis constitute disease entities which may closely resemble pulmonary tuberculosis or other fungus infections and have frequently been misdiagnosed On the other hand *Candida albicans is* fairly *ubiquitous and may* be present in the oral cavity or in the respiratory tract without being the cause of the lung infection under investigation Histoplasmosis caused by *Histoplasma capsulatum* was once thought to be a rare and fatal disease but is now known to be rather widespread and to cause various clinical pictures The natural reservoir of this fungus is the soil where it grows as a saprophyte and man acquires the infection by inhalation of the spores In the respiratory tract *Histoplasma capsulatum* may provoke a variety of pulmonary lesions Most frequently it leads to preponderantly inapparent or subclinical infections and only *rarely does the fungus spread to other organs* and produce a fatal illness Pulmonary histoplasmosis must be considered in the differential diagnosis of a variety of acute and chronic infections of the respiratory tract In chronic active pulmonary histoplasmosis the main differential diagnostic difficulties arise with respect to tuberculosis the lesions cannot be distinguished clinically or radiologically as *cavitation and calcifications are quite common* in both In certain geographic regions many of the cases admitted to sanitaria suffer from histoplasmosis rather than tuberculosis (Furcolow and Brasher 1956) The isolation and the identification of the fungus is essential for the clinical diagnosis Complement fixing antibodies and sensitivity to histoplasmin provide further corroborative evidence *Histoplasma capsulatum* will grow on a variety of media Blood agar containing penicillin and streptomycin Sabouraud s medium and corn meal agar are usually employed for its isolation During the acute febrile stage of pulmonary histoplasmosis blood cultures or cultures of bone marrow are indicated and reveal the frequent transient dissemination of the fungus *Coccidioides immitis* can cause a primary infection of the lungs resembling either atypical pneumonia or tuberculosis if cavitation occurs Coccidioidomycosis can occur as a self limiting disease which probably is more common than is recognized as evidenced by positive skin reaction to coccidioidin The systemic form which as a rule also affects the lungs is a progressive highly fatal disease Growth is easy on Sabouraud s medium but cultural studies are highly dangerous to the personnel and require expert care in order to avoid laboratory *infections Therefore Smith* and others (1956) have advocated the use of less dangerous diagnostic methods such as the complement fixation test and a precipitin test using a coccidioidin antigen The demonstration of specific humoral antibodies provides a conclusive diagnosis According to the authors these tests are successful in 92 per cent of primary coccidioidal cases who are clinically ill In patients with old pulmonary residual lesions such as a cavity the serologic tests are positive only in 60 per cent of cases The skin test carried out with coccidioidin is very specific and should be used in connection with the serologic test Blastomycosis caused by *Blastomyces dermatitidis* frequently starts as a pulmonary lesion resembling pneumonia or tuberculosis and from the primary focus infection may invade other organs leading to systemic disease The diagnosis is made with certainty by the demonstration and the isolation of the fungus Complement fixation tests and skin reactions may be of some aid Other fungi cause lung infections rarely or only as

is no difficulty in isolating and identifying this organism Sputum from chronic infections of the bronchi or from bronchiectasis show a great variety of aerobic and anaerobic microbes It is often difficult to evaluate cultural results in these cases, particularly if the flora consists of organisms generally considered as saprophytes of other organisms found in such specimens little or nothing is known Primary pneumonia due to *Streptococcus pyogenes* is relatively rare but may occur In recent years serious and severe pneumonias due to *Staphylococcus pyogenes* particularly in children, have been seen more often than was previously the case These pneumonias frequently show a fulminating course with abscess formation *in the lungs and generalized septicopyemia* In these cases early bacteriologic diagnosis and testing of sensitivity to various antibiotics of the isolated strains is imperative Many strains of Staphylococcus giving rise to this type of pneumonia are resistant to the majority of available antibiotics and the selection of the most appropriate drug as early as possible in the course of the disease assumes critical importance Pneumonias may also occur as complications of a number of systemic infections The following diseases occasionally give rise to this type of pneumonia typhoid fever plague tularemia and rarely brucellosis and some others In plague the pneumonia may be primary by transmission from man to man Pneumonic processes arising from bronchiectasis aspiration of foreign bodies or as consequence of bronchial obstruction are almost invariably mixed infections Anaerobic as well as aerobic cultures of the sputum or preferably material obtained by *bronchial aspirations must be set up* Fusiforms and spirochetes will be commonly found on stained films and the cultural and microscopic findings have to be considered together for complete evaluation of the case The acute pneumonic form of tuberculosis presents no features characteristic enough to allow differentiation from other pneumonias by clinical criteria and laboratory identification is essential for the final diagnosis This however is a relatively rare form of tuberculosis In subacute and chiefly in chronic conditions of the lungs a number of other considerations apply First and foremost tuberculosis has to be thought of in every case Clinical and x ray findings may be suggestive, but only the demonstration of virulent tubercle bacilli provides definite diagnostic proof By adhering strictly to this criterion serious diagnostic mistakes will be avoided The presence of acid fast bacilli on stained smears must not be relied upon as saprophytic mycobacteria or acid alcohol fast strains of nocardia can occur in sputum or in gastric washings The differentiation of *Mycobacterium tuberculosis* from Nocardia particularly from the acid fast species, *Nocardia asteroides* cannot be made on the basis of stained smears Yet it is of prime importance because these two diseases require different treatments Nocardiosis is a disease which is still not sufficiently understood by *most clinicians Nocardia asteroides is the* most common infecting organism, although other species of Nocardia may be involved It must be realized that the usual concentra*tion methods used for Mycobacterium tuberculosis* may interfere with the viability and the isolation of *Nocardia asteroides* or at least delay its growth The isolation of *Nocardia asteroides* even if the strain proves to be pathogenic for animals, is no valid proof of its being responsible for the pulmonary disease as this organism can reside in the tracheobronchial tree in a purely saprophytic role This is particularly true in patients with bronchiectasis and emphysema Therefore it is often difficult to assess the exact significance of this organism when demonstrated in the sputum only

Fungal infections in general but particularly those of the lungs have received a great deal of attention since World War II Before that time they were regarded as rare and *exotic agents of little practical importance* Probably due to the decrease in bacterial pneumonias particularly lobar pneumonia and the better control of tuberculosis fungous diseases have assumed an increasing importance It has been stated by Smith (1957) that certain fungous infections have increased in number and severity since the introduction of the broad spectrum antibiotics apparently as a result of a temporary disturbance of the normal commensals which usually hold the fungous flora in check Smith also mentions that the use of the cortical steroid hormones has increased the number of infections with fungi of limited pathogenicity and can also

gastro enteritis For definite incrimination of any food and source the strains have to be shown to produce enterotoxin No simple test is available for this purpose and Dolman s or Hammond s (1936) test on kittens has to be used In large outbreaks it is the task of the public health authorities to trace the source However in outbreaks in families the physician should investigate the circumstances and attempt to find the source Phage typing has proved to be an aid in the tracing of food poisoning outbreaks and particular phage types seem to be connected more frequently with staphylococcal food poisoning The third and most serious type of food poisoning is due to the toxin of *Clostridium botulinum* The clinical picture is usually characteristic enough to suggest the cause and laboratory confirmation depends upon the isolation and the identification of the organism by anaerobic methods or more importantly upon the demonstration of the type specific toxin in the food and rarely in the filtrates of bowel contents

Laboratory examination is essential in cases of cholera occurring outside of epidemics and is desirable in cases of mild cholera diarrhea occurring in the course of an epidemic The all important procedure is the isolation and the identification of the vibrio from stool vomitus soiled clothing or bedding Microscopically the typical vibrios are found abundantly in the mucopurulent flakes in characteristic stools but they may be scarce in atypical cases Vibrios grow very rapidly at pH 8 in 1 per cent peptone water (even more dilute solutions may be used to discourage the growth of other organisms) The cultures can then be identified by physiologic tests Blood cultures are useless and titration of immune bodies in the patients serum is of no particular help Pfeiffer s reaction (intraperitoneal bacteriolysis of vibrios by anticholera serum in the guinea pig) is often essential to identification Water from suspected sources must be skimmed carefully from the surface and taken in shaded regions of opened water system such as rivers ponds tanks and wells Slime and mud exposed at low levels of water in the dry season and collected in well shaded situations may prove to be important

Examination of stools for tubercle bacilli is not a commonly carried out procedure but its necessity may arise occasionally Various concentration methods similar to those for sputa are used The finding of tubercle bacilli in the stool is by no means proof of primary intestinal tuberculosis but may be due simply to open lesions in the lungs or the larynx and swallowing of tubercle bacilli containing saliva

In very recent years severe intestinal disturbances have been related to *Candida albicans* This fungus may overgrow the normal commensal flora of the intestinal tract and lead in this region as in the lungs to clinical disease Candidiasis of the intestinal tract is observed in patients during or following antibiotic therapy particularly with the tetracyclines which reduce and inhibit the normal intestinal flora but have no effect upon the yeast Isolation of *Candida albicans* on blood plates or Sabouraud s medium from such stool is as a rule possible as these organisms outnumber the normal flora

All the infections discussed so far represent well established clinical and bacteriologic entities Yet there are a great many patients with symptoms and signs of enteritis of a mild and sometimes even of a severe type in which the most careful bacteriologic studies do not yield any of the recognized intestinal pathogens In some large numbers of Proteus or Pseudomonas or both or organisms of the Paracolobactrum type can be found repeatedly The interpretation and the evaluation of such findings are difficult and require careful study of each individual case To ignore such cultural results by reporting no intestinal pathogens or whatever the accepted formula in an individual laboratory may be is of little help to the patient the physician or to an increase in our knowledge The findings in recent years (Bray 1945 1948 Taylor et al 1949) that certain serotypes of *E coli* may cause enteritis at least in children are proof that our concept of pathogenicity may have to change Only careful studies by the bacteriologist as well as the clinician permit correlation between particular findings and a disease process

The gallbladder and the bile ducts are quite common sites of infection mostly blood borne although spread through the ducts or the lymphatics probably is responsible for some Whether the cultivation of bile obtained by duodenal drainage or during operation provides a complete and accurate index of the

part of a generalized infection The diagnosis can be established more easily by the examination of specimens from the primary lesions

The cultural results from sputum and other specimens from the respiratory tract present the greatest difficulties in interpretation and evaluation with respect to the patient s disease particularly with regard to the chronic *type of infection which usually yields* a great variety of micro organisms This situation is aggravated by the fact that superimposed infections with various fungi are not uncommon in tuberculosis The possibility of simultaneous occurrence of such diseases as tuberculosis histoplasmosis and North American blastomycosis has to be borne in mind Other combinations may occur and require careful study both from the clinical and the laboratory aspects

Exudates from Serous Cavities

All infections affecting the lungs can and do on occasion reach the pleura and lead to pleural effusion or empyema Frequently such exudates provide more satisfactory material for culture than sputum the bacteriologic diagnosis being simpler and more unequivocal The same considerations as outlined for pulmonary infections apply to the study of pleural exudates The advantage of cultivating these exudates consists in the fact that as a rule the interpretation is facilitated by the absence of contaminating organisms The mediastinum and the pericardium can become infected by contiguity or via the lymphatics The management of exudates from other serous cavities joints and peritoneum will be discussed with the appropriate organ systems

Stools Rectal Swabs and Bile

The diagnosis of intestinal infections is important not only for the individual but also *for the whole community as almost all organ*isms involved are potential sources of epidemics Appropriate specimens should be sent to the laboratory as quickly as possible in sterile containers in order to prevent over*growth by contaminants* or even by the normal coliforms which might prevent isolation of the pathogens As enteric fevers usually represent systemic infections a blood culture in the acute phase may give more prompt results than cultures of stools which may not become positive until later in the disease In typhoid fever the cultivation of feces is the *method of choice after the first week of ill*ness The organisms become even more abundant during the second and the third weeks in the feces in untreated cases Although the isolation and the identification of organisms involved in enteric fevers or salmonellosis present no great difficulties the serologic type identification is not possible in most clinical diagnostic laboratories and has to be carried out by specially equipped central laboratories In suspected bacillary dysentery the feces should be sent as soon as possible to the laboratory Within the first 3 days of onset of infection the isolation of the responsible organisms is quite simple From then onward isolation may become increasingly difficult Infections by two or more species or types or by mixed bacterial and protozoal infections occur in tropical regions a fact which has become increasingly important with world wide travel Special selective and differential media for the isolation of enteric pathogens are of great advantage and are available commercially In outbreaks of food poisoning a number of organisms may be implicated and it is important not only to examine the feces but also to obtain immediately samples of the incriminated food as well as of vomitus Food poisoning may be due to different serologic types of Salmonella In these cases the gastro intestinal upsets starts *from 6 to 8 hours after* the consumption of food and usually occur in all members who have partaken of the same food If the specimens are sent immediately to the laboratory isolation of the causative organism *is easily done In such case it is im*portant to identify the organisms as completely as possible for epidemiologic studies and the tracing of the carriers responsible for the out break *Staphylococcus pyogenes* is a frequent *cause of severe food poisoning* the onset is very sudden usually starting 2 to 3 hours or even earlier after the ingestion of contaminated food Only enterotoxin producing strains of *Staphylococcus pyogenes* give rise to this type of disease Staphylococci can be isolated from the stools the vomitus and the food but even the finding of large numbers of staphylococci is only presumptive evidence of their causal relation with a particular outbreak of acute

ous organisms are suspected special enriched media have to be used in addition Urinary carriers of *Salmonella typhosa* or of other salmonella although much less frequent than gallbladder carriers may develop pyelitis and sometimes stones The latter in turn favor invasion by other bacteria resulting in mixed infections Blood stream invasion from the urinary tract especially from ascending pyelitis is a not infrequent complication particularly in childhood Tuberculosis of the urinary tract can be diagnosed only by the isolation of the tubercle bacillus from the urine Although 24 hour specimens of urine provide suitable screening material no diagnostic reliance can be placed on the finding of acid alcohol fast rods in smears as the nonpathogenic smegma bacillus resembles the tubercle bacillus Cultures and animal inoculations will provide the necessary information In order to establish without a doubt which kidney is affected ureteral specimens taken separately from each kidney should be examined As such samples are as a rule very small the most reliable method is inoculation of animals for the final diagnosis Blood borne infections of the kidney may be due to a great variety of organisms including various species of fungi Although in such case the diagnosis is usually made through cultivation of specimens from the primary site on occasion signs and symptoms from the urinary tract may be the most pronounced and urine the most convenient source from which to isolate the organisms

Infections of the genital tract can be divided into two groups those infections contracted by direct contact (venereal diseases and those due either to spread from neighboring organs or occurring as metastatic lesions from primary foci elsewhere Both of these group present their own special problems In the venereal diseases the number of etiologic agents is limited but the demonstration of the microbes may be very difficult at times Gonococci require special methods for their cultivation although easily isolated from pus in acute gonorrhea in the male their isolation from cervical discharges may be unsuccessful unless special precautions are used in the procurement of specimens and several attempts are made Contamination with vaginal secretion must be avoided and the speculum used to visualize the cervix should be sterile and free of any disinfectant or lubricant Even repeatedly negative cultures do not definitely exclude gonococcal infection particularly of the chronic type in the female The successful isolation of gonococci from prostatic gland secretions also depends largely upon the collection of the specimens which must be cultured as soon as possible after they are obtained Although various preserving media are available bedside inoculation gives the most reliable results when delay cannot be avoided The diagnosis in primary syphilitic infection of the genital tract depends upon the demonstration of *Treponema pallidum* by darkfield microscopy in the exudate or in the fluid aspirated from the regional lymph nodes In order to confirm a clinical diagnosis of soft chancre a specimen of discharge should be taken from the somewhat undermined edges of the shallow ulcerations Gram stained films will show the characteristic chain of small gram negative rods which can be isolated on culture by special methods and technics

The Donovan bodies of granuloma inguinale occur within large mononuclear cells fairly deep scrapings containing tissue cells have to be taken from the lesions (Cannefax 1948) Smears are prepared and stained with Wright s stain The Donovan bodies appear as encapsulated coccobacillary or rodlike forms within the mononuclear cells The finding of the organism may be difficult at times and careful search in several smears may be necessary Lymphopathia venereum is caused by one of the large viruses and the diagnosis depends usually upon indirect means skin tests and complement fixation tests It should be realized that quite frequently two or more of these infections may be present simultaneously and the finding of one etiologic agent does not exclude the presence of others For the diagnosis of infections other than venereal of the genital tract the cultivation of suitable specimens and the isolation and the identification of the organisms provide the only reliable procedure For the diagnosis of Trichomonas infections microscopic examination of fresh saline washings of the vagina is the quickest and most suitable method *Candida albicans* a common cause of vaginitis usually can be identified on stained smears and grown and identified on cultures Postpartum or post operative infections of the genital tract are

bacterial flora causing the infection is still debatable It is possible that the bile suppresses organisms sensitive to it, yet capable of producing infection in the wall and that the organisms recovered represent only secondary invaders or only part of a mixed infection *E coli* and enterococci are the organisms most frequently isolated in inflammatory processes of the gallbladder *Salmonella typhosa* or other Salmonella of the enteric fever group are always present in the bile during the active phases of the disease and not infrequently remain in the gallbladder and also in the ducts thus giving rise to a carrier state The presence of these organisms does not necessarily lead to inflammatory changes in the organs, although in many cases the chronic carrier shows signs and symptoms of cholecystitis and gallstones Various other micro organisms are isolated on occasion from the bile but their etiologic relationship to the infection is not always easy to prove

The organisms making up the intestinal flora are probably harmless while confined to the lumen of the gut but may give rise to serious infections in other structures of the abdominal cavity Appendicitis and localized or generalized peritonitis are frequently caused by them blood borne or lymphytic spread infections also occur In the former case a very mixed flora consisting of many anaerobic species as well as of organisms of the colon aerogenes group are found The incidence of peritonitis following abdominal operations has been reduced considerably by the use of sulfonamides and antibiotics Blood borne peritonitis caused by pneumococci is seen mainly in young children but is no longer a frequent type of infection The specific cause in each individual case can be established only by the isolation and the identification of the organisms from the peritoneal exudate Aerobic and anaerobic cultivation is absolutely essential for a complete picture of the flora involved The abdominal form of actinomycosis like that of the lungs is often difficult to diagnose in the early stage The specimens usually contain a very mixed flora and the slow growing *Actinomyces israeli* is difficult to isolate from heavily mixed cultures If no warning is given to the laboratory the diagnosis will be missed because in all probability the cultures will be discarded before this organism had time to grow If the clinical condition is understood, a search for sulfur granules in the exudate facilitates the isolation and the definite diagnosis of actinomycosis

A number of other infections may occur in the abdominal cavity, usually secondary to primary lesions elsewhere chiefly in adjacent structures The types of organism isolated may give an indication of the site of the primary focus Psoas abscesses, subphrenic abscesses, abscesses of the liver, etc, may arise from organs in the abdominal cavity, from bone structures or from the chest In subphrenic abscesses pneumococci streptococci *Hemophilus influenzae* and some others will be found if the primary focus is in the chest *E coli* or a mixture of intestinal organisms will be isolated if the abscess follows perforation of the gut Liver abscesses are either blood borne or arise around infected bile ducts or from infections of neighboring tissues *K pneumoniae* *E coli* *Actinomyces israeli* staphylococci fungi and occasionally other microbes can be isolated from such lesions However the most frequent cause of liver abscesses is *Entamoeba histolytica* Psoas abscesses are due most commonly to *Mycobacterium tuberculosis* by extension from primary tuberculosis of the vertebrae but they may also be caused by pyogenic organisms

Specimens from the Urogenital Tract

Bacterial infections of the urinary tract affect all age groups in both sexes and range in severity from minor discomfort to severe systemic disease The culture of urine is the only means of making a specific diagnosis It is most important to use all necessary precautions to obtain a urine sample uncontaminated with organisms from the skin or the intestinal tract Although reasonably useful specimens of urine can be collected by intermittent voiding and discarding the first running catheter specimens are essential in many cases and always from females Microscopic examination of sediment forms an essential part of every urine examination The organisms giving rise to urinary tract infections are *E coli* Proteus Pseudomonas enterococci *Staphylococcus pyogenes* and others For all these organisms cultivation on routine media will suffice However if *Neisseria gonorrhoeae* or other fastidi

and others however many present no well defined clinical characters Gas gangrene may be due to several species of clostridium which differ in morphologic and physiologic characteristics and also in their specific toxins The appearance of the lesion and the clinical manifestations are characteristically different when caused by a single species of one or another of the clostridia and differ still more markedly when caused by a mixture or when complicated by other kinds of bacteria Infections by anaerobic streptococci are often clinically indistinguishable from clostridial gas gangrene Specimens for bacteriologic examinations must be selected carefully and should contain some *of the tissue as well as the exudate preferably* collected from the area where the infection is progressing The isolation and the exact identification of all species present may be a difficult and time consuming task if the wounds are heavily contaminated Particularly the isolation of *Clostridium tetani* may be exceedingly difficult and even fail in some cases as these organisms are usually present in very small numbers only and strictly localized It is essential to examine deep seated necrotic tissue and especially any extraneous material imbedded in the wound Strict anaerobiasis is an absolute requirement for isolation of *Clostridium tetani* and most other clostridia

Wound infections with *Corynebacterium diphtheriae* are rather uncommon in moderate climates but do occur in tropical or subtropical regions Bacteriologic proof of the presence of toxin producing strains is required for diagnosis and treatment Unless cultures are taken frequently the true nature of such lesions is not recognized until paresis of a limb causes concern The type of flora involved in many accidental wounds depends largely upon the site and the conditions of the infliction or the opportunities for contamination Almost all wounds situated in the lower half of the body are contaminated with coliform organisms some of which like Proteus and *Pseudomonas aeruginosa* may aggravate the condition or maintain the infections after other organisms have been eliminated In all mixed infections great care must be taken to isolate all the organisms present and to guard against overgrowth by certain types which may obscure the presence of actually more important species In most wound infections particularly the most serious ones it is imperative to test all isolated strains for their sensitivity to available antibiotics On the basis of such findings suitable antibiotics or combinations can b chosen for best results In most cases it will be necessary to do repeated cultures unless healing of the wound occurs rapidly The interpretation of all positive findings has to be made in connection with the clinical condition and particularly with regard to the course of the disease under treatment

Materials from infections of the skin and the subcutaneous tissues show special features different from wound infections proper and their discussion including the flora connected *with such infections* seems to be *justified* as a separate group Infections of the skin and the subcutaneous tissues may also occur from within either by contiguity of deeper lesions or via the blood stream and the lymphatics The former type arises more frequently in the course of *chronic infections* (tuberculosis chronic osteomyelitis actinomycosis etc) and is often connected with the formation of sinuses Infections from without are usually connected with the hair follicles and the sebaceous glands which provide a nidus for the development of focal injury eventually resulting in furunculosis or other lesions They also occur through breaks of the epithelial layer of the skin and rarely through direct infection of the superficial subcutaneous tissues through biting insects *Staphylococcus pyogenes* is the organism most commonly involved in infections of the skin and the subcutaneous tissues Such infections may occur under a variety of clinical pictures (furuncles or boils carbuncles cellulitis etc) In all cases of *Staphylococcus pyogenes* infections the main concern is to prevent spread from the superficial areas into the blood stream and the lymphatics by effective treatment It is the task of the laboratory to determine the most suitable antibacterial agent against the particular strain *Streptococcus pyogenes* infections of the skin also occur in a variety of clinical forms Erysipelas may develop in accidental or operative wounds or follow trivial hardly noticeable pricks or scratches In children a common form of *Streptococcus pyogenes* infection affecting the most superficial layers of the skin is impetigo contagiosa Lymphangitis and regional lymphadenitis are char

obvious indications for bacteriologic examinations anaerobic cultures never should be omitted

Materials from Bone and Joint Infections

The organism most commonly found in infections of the bone is *Staphylococcus pyogenes* giving rise to hematogenous osteomyelitis which occurs *mainly during the first two* decades of life and more often in early childhood The structure of the bones and the arrangements of the blood vessels are believed by many to be the chief factors in the localization of infections on the diaphyseal side of the epiphysis (Wilensky 1934) In some cases *it is periosteal* The primary lesion on the skin or the buccopharyngeal mucous membranes and the nose is often insignificant, even the bacteremic stage may pass unnoticed and the first clinical manifestations of the infection may arise from the localization in the bone The onset may be abrupt or insidious Present day treatment facilities usually can achieve cure or arrest of the process in the early stages chronic staphylococcal osteomyelitis has become a rarity In children under 2 years of age osteomyelitis can also but rarely be caused by *Streptococcus pyogenes* Other organisms may localize in the bones as a complication of systemic infection Suppurative osteomyelitis of the ribs is seen as a complication of enteric fevers pure cultures of typhoid bacilli or other Salmonella can be isolated from such abscesses Metastatic lesions in the bones can also occur as part of a generalized spread in severe systemic infections often during the terminal stages Infections of the bone and the periosteum following compound fractures and other severe injuries to the bone or arising by contiguity from infections of the surrounding tissues are caused by a variety of organisms and frequently by mixtures These cases require special attention and often present serious treatment problems The bone and the periosteum may also become involved in the course of chronic bacterial or fungous infections from adjacent tissues for instance tuberculosis actinomycosis blastomycosis Attention has been drawn in recent years to Brucella infections of the bone and it has been claimed that isolation of the organism may be easier from these lesions than from the blood stream (Coventry et al 1949) The choice of culture media and the methods of incubation *depend upon the clinical provisional diagnosis*

Infections of the joints can also be either blood borne or due to spread from lesions of the bone or the soft tissues or directly introduced through penetrating wounds The most frequent causes of blood borne infections are *the gonococcus and the pneumococcus In a* fairly high percentage of meningococcal infections the joints may also be affected, and meningococci can be isolated from the joint fluids In the non blood borne infections of the joints different species of bacteria may be involved depending upon the flora of the primary lesion from which the infection originates *Mycobacterium tuberculosis* causes a chronic destructive infection mainly of the large articulations—the hip the knee and the vertebrae The large diverse group of cases of nonpurulent arthritis creates many diagnostic problems and only rarely can the bacteriologist contribute to their solution In cases of Reiter s syndrome attempts should be made to isolate organisms of the pleuropneumonia group (Mycoplasma PPLO) However the exact role and the importance of these organisms is not clearly established even for this form of arthritis (Butas 1957 Edward 1955 Freundt 1955)

Specimens from Wound Infections

A wide variety of *micro organisms singly* or in mixtures, can be isolated The available amounts of exudate may be small and can be collected only on sterile cotton swabs or a platinum loop If large quantities of pus or serous exudate can be obtained a good representative amount should be collected but it is *well to remember that scraping from the wall* of an abscess or a sinus is more suitable and more reliable than old static pus from the lumen The selection of specimens from a particular spot is often most valuable in infected wounds especially when secondary contamination prevails and may obscure the original *primary infection The choice of culture media* and the type and the variety of examinations on the material must be guided by the clinical picture Some wound infections lead to recognized clinical entities such as gas gangrene

however it may be permissible to emphasize the complexity of the situation by a few examples The isolation of *Salmonella typhosa* from feces may indicate either active disease or a carrier state The bacteriologist cannot distinguish between the two on the basis of cultural results Other tests may help but it is primarily the duty of the physician to make the decision Similar problems arise in other carrier conditions and may lead to serious confusion and loss of valuable time Another serious source of error may originate from laboratory reports based on inadequate studies The following examples may serve as illustrations the diagnosis of tuberculosis based on the finding of acid alcohol fast rods on microscopic examination the reporting of organisms as hemolytic streptococci without further exact definition which can be misleading as Lancefield group B streptococci in the mouth may come from a drink of milk just prior to the taking of the throat swab No diagnostic significance can be attached to the isolation of a number of bacteria known to cause disease chiefly or solely by their toxins unless proof of toxin production of the particular strain can be established Other difficulties arise through changes in concepts of pathogenicity Mention has already been made of certain serologic types of *E coli* recently shown to be associated with outbreaks of gastro enteritis particularly in infants whereas *E coli* used to be considered as part of the normal intestinal flora The changes in regional flora brought about by the wide use of antibiotics required reorientation in the interpretation of findings in many instances the true importance of these changes is as yet to be recognized The greatest challenge perhaps to the diagnostic laboratory is not to miss a double infection as for example tuberculosis and a fungous infection occurring simultaneously in the same patient The few examples quoted here should give the student an appreciation of the difficulties with which the bacteriologist may be confronted and indicate to him the need of close co operation

SPECIAL AND INDIRECT METHODS OF DIAGNOSIS

Whereas the isolation and the identification of the etiologic agent is the most satisfactory method of diagnosis unfortunately it is not always possible Isolation of the causative organism may fail when healing has taken place or when the organisms have been suppressed by treatment or when culture methods are not effective enough to permit growth from very small numbers of bacteria It may also fail if special requirements of the organisms have not been taken into consideration or when no methods are available for the successful cultivation of a bacterium as is the case with *Mycobacterium leprae* and probably some others Under these conditions it is important to test for the antibodies formed during infection as a means of indirect diagnosis One such method is titration of the patient s serum for agglutinins against known cultures This has been valuable in a fair number of infectious diseases The classic example is the Widal test introduced originally for the diagnosis of typhoid fever after the second week of disease This method is based upon the determination of the greatest dilution of the patient s serum which will cause agglutination of a known bacterial antigen prepared in a standard way

Usually macroscopic methods carried out with reasonable measurable volumes in test tubes are more satisfactory The other methods are desirable only if very small quantities of material are available Agglutination titers vary considerably according to the technic used and comparisons can be made only between tests carried out by the same technic Titers also differ between tests because of unexplained variation in agglutinability of different cultures of the same strain The more exact determination of agglutinin nitrogen is too laborious for routine diagnosis The specificity of the reaction is relied on for its diagnostic value However in certain specific infections because of cross reaction with common antigens special knowledge of the antigenic constitution of the organisms involved is required for interpretation It is always advisable to construct a curve of agglutination titers determined by 2 or 3 tests performed at intervals of a few days when a rising curve of specific antibody titer allows of more certain interpretation especially in vaccinated individuals

The agglutination test is useful in the diagnosis of typhoid fever salmonella infections brucellosis tularemia leptospirosis typhus and a few others It is of very doubtful value

acteristic clinical manifestations of Streptococcal infections The now less common, deeper and more dangerous cellulitis and phlegmons involving fascial planes must be remembered The organism can be easily isolated and identified and most strains of *Streptococcus pyogenes* are highly sensitive to sulfonamides and most antibiotics Infections with *Bacillus anthracis* because of their rarity, may be easily overlooked Cultures of this organism have to be handled with great care and animals used for inoculation must be strictly isolated as the spores are highly resistant, and there is great danger of serious regional contamination The laboratory should be given fair warning if such an infection or any other highly contagious infection is suspected in a patient often indicated by the patient s occupation Infections due to animal bites or to scratches suffered during skinning or dressing may be caused by a number of different types of organisms Pasteurella is a common cause of infections after cat bites and occasionally dog bites (Allin 1942) Infections caused by *Erysipelothrix rhusiopathiae* are acquired mainly as occupational infections by butchers fishmongers, housewives etc The isolation of this organism often requires special conditions of cultivation (Sneath et al 1951) *Pasteurella tularense* infections are contracted through handling of infected animals chiefly rabbits or through the bites of tabanids or ticks or through bites by animals which carry the organisms in their mouth after feeding on infected rabbits (dogs cats etc) For the cultivation of *Pasteurella tularense* special media are required and co operation between the physician or the surgeon in charge of the case and the laboratory is absolutely essential Infections of the skin and the subcutaneous tissue by *Mycobacterium tuberculosis* may appear under various clinical pictures almost invariably causing differential diagnostic problems which can be solved only by the isolation of the organism from the lesions In subacute or chronic infections of the skin and the subcutaneous tissue the bacteriologist has to be on guard against surface contaminants and secondary pyogenic infections obscuring the more important primary cause for example *Mycobacterium tuberculosis Sporotrichum schenkii Blastomyces dermatitidis Nocardia* etc These various types of organisms should be considered, and appropriate media and methods of cultivation used

Already it has been pointed out in a different connection that fungous infections can no longer be considered as rarities Infections with dermatophytes present their own special problems, and early recognition can prevent stubborn and costly epidemics and individual hardships Material for microscopic examination and cultures consisting of hair scales etc, should be collected from all cases suspected of infections with dermatophytes before any treatment is started, otherwise isolation of the fungus is made more difficult, and secondary infections by bacteria may complicate the situation The exact identification of the species of the dermatophyte involved is in some cases essential for rational treatment The growth requirements and characteristic microscopic and colonial features of the dermatophytes, as well as of the fungi giving rise to systemic disease are discussed in detail in the chapter on mycotic infections The interpretation and the evaluation of findings from cultures of the skin and the subcutaneous tissues present as many (or more) difficulties as those from the respiratory tract or the intestinal tract Careful consideration must be given to the clinical picture and course before the etiologic relationship between an isolated organism and the disease process under investigation is accepted Many of the organisms isolated may be harmless saprophytes accidentally present on the skin others although recognized pathogens may be present as secondary invaders and not be involved in the primary infection Erroneous interpretation of findings may have serious consequences for the patient

GENERAL REMARKS ON THE INTERPRETATION AND THE EVALUATION OF CULTURAL FINDINGS

A premise for the successful performance of the bacteriologist is a clear knowledge and understanding of the bacteria which are commonly found regionally in the normal human body No detailed account need be given here and the reader should refer to the chapter on bacteria indigenous to man for a full understanding of the problems and the difficulties which the commensal flora poses in the interpretation of cultural results In addition

is referred to special books on this subject In some diseases of bacterial fungal and protozoal origin complement fixation tests are used as a diagnostic aid but the results can be evaluated only in conjunction with clinical and other bacteriologic studies In acute uncomplicated gonococcal infection of short duration the test is almost always negative and so does not exclude gonorrhea but in long standing infections particularly accompanied by complication (epididymitis salpingitis arthritis etc) a positive reaction occurs and is of diagnostic significance However a positive test is not necessarily indicative of active disease as it may remain positive for months or even years after complete cure

The demonstration of complement fixing antibodies is of diagnostic and prognostic value in certain fungous infections In North American and South American blastomycosis positive complement fixation tests indicate systemic progressive infection and the presence of complement fixing or precipitating antibodies in coccidioidal granuloma has similar significance (Conant 1948) Complement fixation tests are used occasionally with limited success in whooping cough glanders trichinosis echinococcus infection and malaria (Mayer and Heidelberger 1946)

Precipitation tests are used only occasionally as a diagnotic method except in syphilis In this disease the various types of precipitation or flocculation tests are used more widely than even complement fixation because of their relative simplicity Precipitin reactions have important applications in forensic medicine and in public health for the identification of species origin of blood and tissues and of adulteration of food products They also allow the identification of blood sucking insect meals

Skin Tests

The intracutaneous injection of suitable specific antigens is used widely in diagnosis it depends on the local inflammatory response to the reaction between antigen and antibody With toxic antigens which of themselves induce injury and inflammation the presence of antibody is determined by their neutralization and the consequent lack of erythema or other manifestations In the latter instance some degree of quantitative estimate can be made by the amount of toxin which fails to cause reaction on injection It will suffice to enumerate here the most commonly used skin tests

The Schick test is used to indicate blood levels of diphtheria antitoxin (1/250 unit per ml of serum is sufficient to protect against the disease)

The brucellergin test carried out with the nucleoprotein fraction of Brucella (Huddleson 1943) or the injection of Brucella vaccine is employed for the diagnosis of brucellosis The reaction is read after from 48 to 72 hours Erythema with induration of at least 1 cm in diameter represents a positive test evaluation should be done in combination with results of other tests for brucellosis

The tuberculin test with Koch s Old Tuberculin or the purified protein derivative (P P D) is used as a diagnostic aid in suspected cases of tuberculosis It indicates an allergy to the protein fraction of the organism but does not distinguish between past and present infection

The skin test for tularemia uses bacterial vaccine Foshay (1940) claims positive results in 92 out of 100 cases in the first week of disease

The Ito Reenstierna reaction (1913 1924) for the diagnosis of chancroid uses *Hemophilus ducreyi* vaccine The test remains positive for years after recovery a fact which has to be considered in its evaluation

Diagnostic skin tests are also used in several fungous diseases The Histoplasmin skin test is used both as a diagnostic aid and as an epidemiologic tool (Loosli 1957) A positive reaction has the same significance as a positive tuberculin test Sensitivity develops during the third or fourth week after infection and persists for many years Although occasionally individuals reacting strongly to histoplasmin may react to blastomycin and coccidioidin this does not invalidate the use fulness of the test (Furcolow 1948) For the demonstration of cutaneous sensitivity in blastomycosis a vaccine of the killed yeast phase of the fungus or a culture filtrate called blastomycin is used The interpretation of this test is difficult inasmuch as cross reactions with histoplasmin and coccidioidin occur and other misleading results have been reported (Smith 1955) The coccidioidin skin test is carried out with a culture filtrate sensitivity

in shigella dysenteries Agglutination of "H" and "O" antigens of typhoid and paratyphoid in serum dilutions over 1 80 is considered to have diagonstic significance unless vaccination has taken place recently The duration of illness is an important factor and it may be necessary to repeat the test in order to observe a rise or a fall in titer before any final diagnostic conclusions can be drawn

In brucellosis the agglutination test is of even greater importance as an aid in diagnosis because the isolation of *Brucella abortus* is often unsuccessful The heat killed phenolized bacterial suspension must be made from a smooth strain and must be tested for its agglutinability by known positive and negative sera (W H O brucellosis centers supply such antigens) The test should be carried with a wide range of serum dilutions because "prozone" reactions are quite common The possible occurrence of "blocking" type antibodies should be kept in mind It is very important that blood for the test be drawn before skin tests are performed or treatment with vaccine begun as either of these procedures may cause a false positive reaction Cross reactions may occur with cases of tularemia and with sera of patients who were vaccinated against cholera In the interpretation of the results of Brucella agglutination tests occupational groups which have a high rate of exposure—butchers meat packers dairymen farmers veterinarians and drinkers of raw milk—have to be taken into account In these a positive agglutination of brucella in a relatively low serum dilution of 1 80 or 1 100 may indicate only some past or subclinical infection (Evans et al 1938 Huddleson 1943) while the same titer in other groups is suggestive though not proof of active infection Usually the results of other tests have to be considered as well (see interpretation of tests by Huddleson 1943), because a negative agglutination test does not exclude active infection with brucella As further aid in diagnosis of brucellosis the opsonocytophagic test is helpful but it requires considerable experience

If the isolation of *Pasteurella tularense* is unsuccessful or cannot be attempted for lack of facilities the agglutination test can aid in the diagnosis of tularemia Antibodies appear during the second week and show a rise in titer during the third week and up to the fifth week This rise in titer is considered as being reliable proof of tularemia Agglutinins may persist for the rest of the patients life (Ransmeier and Ewing 1941)

Serologic methods have wide application and provide a convenient tool in the diagnosis of leptospiral infections However they are of no value during the most acute phase of the disease, since agglutinins rarely appear before the tenth day The agglutination lysis test (Schuffner, 1927) or its modification (Gardner 1947) requires living viable cultures The maintenance of large numbers of serologic types is not always feasible In recent years satisfactory results have been obtained by the use of killed (formalinized) leptospiral suspensions in a microscopic agglutination test (Ward et al 1956) Other serologic methods have been proposed recently

The *Weil Felix* reaction (1916) for the diagnosis of rickettsial diseases is mentioned here because bacterial suspensions (Proteus) are used as antigens in the test The antigens must be prepared from reliable strains of Proteus OX19 OXK and OX2 and the technic of the test is the same as for other bacterial agglutination tests

Agglutination of other than bacterial antigens is used in some diseases as a diagnostic test One example is the agglutination of sheep red blood cells by the serum of patients with infectious mononucleosis (Paul and Bunnell 1932) Sera from healthy people or those suffering from serum sickness quite frequently agglutinate sheep red cells but usually in low titer (1 40 or 1 60) It is important to distinguish these agglutinins from the antibodies which develop in the course of infectious mononucleosis Bailey and Raffel (1935) found that this could be done by absorption tests A different type of hemagglutination reaction has been developed in recent years Specific antigens are adsorbed onto erythrocytes of various species and then used for the detection of antibodies (Middlebrook and Dubos 1948 Neter et al 1956)

Complement Fixation Tests

These tests are very useful in the diagnosis of many diseases The classic example is the Wassermann test and its modification for the diagnosis of syphilis It is beyond our scope to go into details of technic, and the reader

etc In principle two methods are available (1) a serial dilution method of the agent in a suitable culture fluid and (2) the paper disk or tablet method in which certain known but limited concentrations of the chemotherapeutic agent are contained in the disk or tablet which are placed on the surface of a solid culture medium inoculated with the organism under examination Results are interpreted on the basis of growth inhibition in both methods The serial dilution method provides a more quantitative evaluation but for most purposes the zones of inhibition around the disks or tablets will suffice provided that the preparations used are uniform and constant The lack of accuracy of the latter method is compensated by the greater speed with which results are obtained Control of treatment is afforded by repeat cultures which may reveal resistant forms of the same organism or other bacteria which have taken advantage of the altered conditions

Estimations of Antibiotic Levels in Various Body Fluids

In the majority of cases under treatment routine antibiotic levels are no longer requested but under special conditions it may be desirable to have accurate estimations on an individual patient In essence the same methods are used as for the sensitivity testing Various dilutions of the body fluids are tested against a standard sensitive organism and compared with known concentrations of a control set Most of the fluids have to be diluted within certain ranges which depend on the amount of antibiotic received by the patient and on the time elapsed between the last injection and the taking of the specimen The form of treatment (local intravenous intramuscular intrathecal etc) has also a definite effect For instance it is important for the laboratory to know if an antibiotic was given intravenously or intrathecally if its content in the spinal fluid is to be estmiated All this information should be furnished by the clinician to the laboratory which can then make appropriate arrangements All fluids to be studied for drug concentration should be taken under aseptic conditions into sterile containers because all the biologic methods are based on the inhibitory action of the fluids on standard test organisms therefore their results are confused by contamination

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is developed by the third or fourth week following infection. The test is considered as being very specific if dilutions of 1 100 or 1 1 000 are used. In disseminated disease skin reactivity is frequently lost and this is a bad prognostic sign. Intradermal reactions are of diagnostic value in some virus diseases, e g, the Frei test; they have also been used as a guide in immune serum therapy, e g, pneumonia. Skin tests require considerable care in performance and judgment in their interpretation.

Darkfield Microscopy

This method is particularly helpful in the diagnosis of spirochetal diseases. It is carried out either directly on material from the patient or indirectly on material from inoculated animals. Darkfield is the method of choice in the diagonsis of syphilis particularly in the early stages before serologic tests are of any value. The lymph node puncture is preferable to direct examination of lesions in the mouth because certain oral spirochetes are morphologically indistinguishable from *Treponema pallidum*.

Darkfield examination of blood reveals the Borrelia during a febrile attack of relapsing fever; when few in number their violent motion aids in finding them. Susceptible animals injected with the blood will show large numbers of Borrelia when their own blood is examined later in the darkfield, but this is not effective with all species, e g, *Borrelia recurrentis* has to be passed through the monkey before it will infect mice or other laboratory animals.

In rat bite fever the blood of the inoculated guinea pig should be examined daily under darkfield from the fifth day after inoculation and not infrequently blind passage is necessary. Few other organisms are as rapidly motile as *Spirillum minus*. They can also be stained with gentian violet.

Leptospira are practically never found in the blood or the urine of patients by direct examination but they can be demonstrated by darkground illumination in the blood of infected guinea pigs or in suspensions of the suprarenals, the liver or the kidneys after the animal's death. Fresh fluids should be examined whenever possible to see characteristic movement of the organism.

Coles method (1915) is a very elegant and helpful means of finding spirochetes, especially when they are scanty in the fluid to be examined. Thick films are made and dried in air as quickly as possible, blood is dehemoglobinized with distilled water after drying and is fixed with alcohol. The preparation is stained with any Romanowsky stain (Leishman, Wright, Giemsa, etc) and examined under darkfield illumination. The spirochetes often do not stain well enough to be seen by direct transmitted light, but they are brilliantly opalescent in the darkfield with the colors of a fire opal and are seen very easily.

PROBLEMS AND TESTS IN RELATION TO SULFONAMIDES AND ANTIBIOTICS

The widespread use of sulfonamides and antibiotics has created a number of new problems for the diagnostic laboratory, affecting technical methods as well as the interpretation of results. The cultivation of specimens collected after the patient has been treated with one or frequently with several of these substances may lead to very confusing results with little diagnostic validity with regard to the primary infective agent. The addition of inhibitory substances to culture media (para aminobenzoic acid to neutralize sulfa drugs, penicillinase or clarase to antagonize penicillin, cysteine against streptomycin) is by no means a satisfactory substitute for specimens obtained before any drugs have been administered.

Very early and successful antibiotic therapy of certain infections (e g, typhoid) may interfere with the diagnostic significance of serology tests.

Susceptibility Tests

The testing of bacteria for their sensitivity to antibiotics has become a routine procedure in the diagnostic laboratory. It is more and more appreciated that the usual sensitivity tests provide only a qualitative guide in the choice of an antibiotic. Agreement between in vitro tests and clinical results is only approximate, and concentrations of antibiotics insufficient to prevent growth in the test tube may be effective in the body. The end points obtained in the sensitivity testing are dependent upon a number of factors—size of inoculum, length of time of incubation, media

Appendix

MATERIALS AND METHODS

Media

Media are classified roughly either on the basis of their physical state into solid liquid and semi solid or more often on the basis of their use and functions On this latter basis media tend to be classified into basal media media primarily designed for biochemical tests or the recognition of physiological characteristics of individual organisms enriched media differential media and selective media Media have also been divided on the basis of their chemical composition into synthetic non synthetic and semi synthetic media Up to now purely synthetic media of completely known composition have relatively few applications in medical bacteriology

The basis of most media used in the diagnostic laboratory is an infusion of meat to which is added peptone and salts in known quantities To this base agar may be added to get a solid surface or form a jelly of desired consistency The great advantage of agar is that it melts at 100 C and sets at about 45 C —a temperature which allows the mixing of heat labile substances and of living bacteria without fear of inactivation Agar remains a firm jelly at most required incubation temperatures To the basal media which permit the growth of a variety of non fastidious organisms various substances are often added to promote growth of fastidious species (blood serum ascitic fluid fresh tissues etc) Such media are called enriched media but at the same time they permit also certain differentiation by the changes which bacterial growth produces in them—for instance hemolysis or greening of red blood cells Substances can also be added to either basal or enriched media to inhibit selectively the growth of unwanted species while allowing growth of others (Brilliant green tetrathionate sodium sulphite and many others) Reducing substances may be added to produce an environment of low oxidation reduction potential (sodium thioglycollate cysteine etc) Yet other substances known to be tolerated or not tolerated or altered by the organisms being studied are added for differentiation purposes Many media are designed primarily for the detection of by products of metabolism acid gas indole liquefaction of gelatin reaction on litmus milk e g stormy fermentation coagulation digestion of casein reduction of indicator etc In order to make use of these functional applications of culture media it is imperative that only the bacteria present in the material to be studied be allowed to grow The most convenient and safe method of sterilization is steam under pressure in the autoclave and this is used wherever heat and moisture are not injurious to the material Where heat under pressure is undesirable sterilization can be obtained by filtration Depending on the materials to be sterilized various types of filters are used (Berkefeld Chamberland sintered glass or membrane filters) It is not intended here to give even a brief formulary of the hundreds of different media that have been described On this content the majority of diagnostic laboratories uses commercially prepared media especially as basal media to which various ingredients are added as required

Temperature and Time of Incubation

In addition to food requirements there are other important factors to be considered for optimum bacterial growth one of them is temperature Most pathogenic bacteria grow at about 37 C and this temperature has to be maintained uniformly for a suitable time Incubators at higher and lower temperatures should also be available because temperature tolerance provides differentiating criteria for certain species Some bacteria have a relatively wide range of tolerance while others grow only within very narrow limits Improper temperature cannot be compensated for by increasing the time of incubation A great many pathogenic bacteria develop visible colonies after 18 24 hours of incubation at optimum temperature but there are some notable exceptions which have to be considered to avoid missing important organisms *Mycobacterium tuberculosis* on conventional media requires at least several days but may require weeks before growth is obtained Somewhat quicker results are obtained by using a medium originally described by Dubos et al (1947) and its various modifications Considerably more

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container 0 24 Gm of sodium carbonate and 4 cc of 10 per cent sulfuric acid for each liter capacity of the jar used when the chemical reaction begins to subside seal down the lid and incubate Special methods have been devised to increase the CO tension inside individual culture bottles (Huddleson 1943 Shaughnessy 1939)

A SIMPLIFIED GUIDE TO THE PROVISIONAL RECOGNITION OF COMMON GROUPS OF BACTERIA

The purpose of the following listing of selected characters is to enable inexperienced workers to recognize the main groups of commonly found organisms Once such a group has been determined reference can be made to the appropriate chapter of this book and to tables and descriptions in books such as Bergey s Manual of Determinative Bacteriology for final identification This list is not a determinative key but can serve as a first step in practical procedure Only commonly found bacteria have been included less common and unusual forms have been left out for the sake of simplicity

I Aerobic cultures

1 Gram positive cocci

A Arranged in clusters form large pigmented opaque colonies *Staphylococcus* Distinguish pathogenic from nonpathogenic species by plasma coagulase and biochemical characters

B Short and long chains and even pairs in pus—*Streptococcus*

a Pyogenes Group distinguish by hemolysis Lancefield grouping Does not grow at 45 C

b Viridans Group greening of blood not soluble in bile do not ferment inulin grow at 45 C and not at 10 C

c Enterococcus Group some hemolytic and some no action on blood grow at 10 C and 45 C and survive 60 C for 30 minutes Grow in 1 per cent methylene blue and in 6 5 per cent NaCl All Lancefield Group D

d Pneumococcus greening of blood (Hemolysis in anaerobic culture) Soluble in bile well marked capsule ferment inulin Typing by capsule swelling with specific antiserum

2 Gram negative cocci mostly in pairs

A *Neisseria meningitidis* requires enriched media ferments dextrose and maltose with acid production saccharose lactose and levulose not fermented Agglutination by type antimeningococcus serum and if not available by polyvalent serum

B *Neisseria flavescens* requires enriched media yellow colony no carbohydrates fermented no agglutination with antimeningococcus serum

C *Neisseria gonorrhoeae* requires specially enriched media ferments dextrose only Positive oxidase test helps isolation from mixed cultures from cervix The alkali solubility test described by Cantor et al (1942) is important No agglutination with meningococcus sera

D *Neisseria catarrhalis* grows well on ordinary culture media no fermentation of carbohydrates

E Pharyngeal group (*N sicca N flava N perflava N subflava*) grow well on ordinary culture media pigment production is best on Loeffler s slant distinguished by biochemical reactions

3 Gram negative rods

A Grow freely on ordinary media ferment carbohydrates no capsule Motile and nonmotile (peritrichous flagella)

a Lactose positive—Coli aerogenes group

b Lactose negative—Salmonella Shigella groups

Differentiate members of the groups by biochemical tests and specific antisera

B Some requirement of special media and conditions no fermentation of carbohydrates no capsule nonmotile—*Brucella*

rapid results can be obtained by the use of slide culture methods (Reed and Morganti 1956) Other organisms, brucella, actinomyces and the various fungus species also require prolonged times of incubation Between the fast growing organisms and those that need weeks for development there are species exhibiting a wide range of requirements of incubation time This is often characteristic either for the production of certain reaction or for the development of growth Thus there is a definite time and a definite temperature which are best for the production of diphtheria toxin and these differ from those required for the production of tetanus toxin or staphylococcus toxin Considerations as to temperature and time of incubation apply therefore not only to the primary isolation of organisms but even more so to the various characteristic reactions—by means of which they can be differentiated

Atmosphere

Only the practical applications will be emphasized here It is a sound rule to set up all cultures in duplicate and to incubate one set aerobically and the other anaerobically Various methods are available for the production of anaerobic conditions Brewer s (1940) thioglycollate medium permits the growth of anaerobes under the usual technic of incubation used for aerobes and can therefore be used by small laboratories which have no special anaerobic equipment Other media containing reducing substances have been recommended for the growth of anaerobes under the routine conditions of incubation Thus glucose has long been known to create partial anaerobic conditions by its reducing action The addition of a small amount of agar (0 1% to 0 3%) produces slightly viscid media which provide various degrees of anaerobiosis (Falk et al , 1939) Cysteine (0 3%) added to agar or infusion broth acts as a reducing agent All these methods have the disadvantage that they do not provide adequately for the growth of surface colonies and when dealing with mixed cultures, necessitate replating in order to permit isolation of pure cultures Special culture dishes have been designed in an attempt to avoid this trouble (Brewer 1942 Spray 1931 and 1936) So-called deep shake agar cultures are also used and with some experience colonial morphology can be differentiated Absorption of oxygen by chemicals does not require any special apparatus Any jar with a well fitting lid can be used A dish containing pyrogallic acid is put in the bottom of the jar—the culture plates or tubes are put on a wire rack above the dish A 5 or 10 per cent sodium hydrate solution is added to the pyrogallic acid and the jar is quickly sealed with a suitable lute The absorption of CO_2 by the alkali may at times interfere with the growth of organisms requiring it Cultures of various bacteria which consume oxygen rapidly or a burning candle can also be added to sealed jars to remove oxygen from the atmosphere

Specially constructed jars for the cultivation of anaerobes are based on the combination of oxygen with hydrogen by catalytic action The McIntosh and Fildes (1921) jar uses palladinized asbestos as catalyst in a small wire cage surrounding a small electric bulb which provides heat to start the reaction The jars are made either of metal or glass and the lids have to be well fitted and provided with terminals to the heating element and with stop cocks to introduce the hydrogen the pressure is controlled by means of manometer These jars permit the growth of all types of cultures and pure culture isolation from surface colonies is as easy as with aerobes They afford complete anaerobiosis which is essential for some organisms Various modifications of the same general principles are in use The modification by Brewer and Brown (1938) and Brewer (1939) permit the use of illuminating gas Chromium sulfuric acid mixture in the bottom of an acid resistant jar has been recommended by Rosenthal (1937) The method is cheap and simple 15 per cent solution of sulfuric acid (100 cc per liter capacity) and 5 Gm of chromium metal (per liter) are put in the bottom of a jar The plates and tubes supported on a stand are put in and the jar is sealed The lid must contain a gas outlet which is closed after the vigorous formation of hydrogen has subsided Whatever method is used a tube of dextrose broth containing a small amount of methylene blue should be placed in the jar to test anaerobiosis by the reduction of the dye

Increased concentrations of carbon dioxide is required by certain organisms especially on first isolation e g Brucella abortus Neisseria gonorrhoeae Staphylococcus pyogenes produces its specific toxin in much higher quantities in an increased CO_2 tension

Any of the jar used for anaerobic cultivation can be used for the carbon dioxide method The desired amount of air is evacuated and replaced by CO_2 from a tank An atmosphere containing 10 to 18 per cent CO_2 is usually required Another method to obtain increased CO_2 tension is the following place in an open

tive and esculin not hydrolyzed—*Erysipelothrix* (*rhusiopathiae*)

C Motile short rods catalase positive and esculin hydrolyzed—*Listeria monocytogenes*

D Nonmotile beaded staining acid fast—*Mycobacterium*

a *Mycobacterium tuberculosis hominis* and *bo is* are the 2 types of importance Distinguish from saprophytic members by cultural characters and pathogenicity tests

b *Mycobacterium leprae* cannot be grown in culture not pathogenic for laboratory animals acid fast stains of smears of nasal secretions or skin lesions show vast numbers cramming the so-called lepra cells

E Spores formed usually without distortion of cell—*Bacillus* One species of medical importance *Bacillus anthracis* grows well on simple culture media characteristic colonies on agar nonmotile causes fatal septicemia in laboratory animals capsul formed in animal body

II Anaerobic cultures

1 Gram positive cocci

A Occurring mainly in clusters but also in pairs—anaerobic Staphylococcus (Peptococcus)

B Mainly in pairs and chains—anaerobic Streptococcus (Peptostreptococcus) Identification by growth characters and biochemical reactions—see special chapters and Bergey s Manual

2 Gram negative cocci

In irregular masses very small cocci Veillonella both types often found in tooth abscesses and in infections of genito urinary tract For identification and differentiation—see Bergey s Manual

3 Gram negative rods

A Motile or nonmotile of varying sizes and shapes non spore forming often foul smelling—*Bacteroides*

B Large pointed ends effuse colonies and difficult to grow—*Fusiformis* (*Fusobacterium*)

4 Gram staining variable true mycelium produced true branching non acid fast—*Actinomyces* Identified by cultural characters (Slow growing colonies on solid media dry crumbly resembling tubercle colonies in fluid medium granules adherent to walls of tube Unstained preparation of crushed granule shows typical clubs)

5 Gram positive rods motile and non motile forming endospores which distort the cell some species microaerophilic—*Clostridium* Many members of this group are saprophytes but some are highly pathogenic for man and animals Differentiation by biochemical reactions and pathogenicity tests and specific toxins

APPENDIX REFERENCES

(References mentioned in the text but not listed in this bibliography will be found in the second edition of this textbook)

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Spray R S 1936 Semisolid media for cultivation and identification of the sporulating anaerobes J Bact 3 135 155

Members of this group have to be differentiated by CO_2 requirement H_2S production, dye inhibition test and agglutinin absorption tests

C No growth or poor growth without special growth factors in the media Capsule variable nonmotile—*Hemophilus* and *Moraxella*

a *H influenzae* shows characteristic satellitism along staphylococcus streak or other colonies no growth on plain agar encapsulated strains (mainly from C S F) identified by type specific antisera

b *H pertussis* shows characteristic colonies on Bordet Gengou medium agglutination by specific antiserum

c *H ducreyi* cultivation rarely done for diagnostic purposes (clotted rabbit sheep or human blood heated at 55° C for 15 minutes is inoculated with scrapings from suspected lesions or pus from lymph nodes and incubated at 37° C for 24 hours Gram stained films of the serum show small gram negative bacilli often in chains)

d *H parainfluenzae* grows on plain agar along a staphylococcus streak or other colonies providing V factor

e *Moraxella lacunata* from angular conjunctivitis no growth on blood agar characteristic pitting on Loeffler s serum slant typical diplobacillus

f *Moraxella liquefaciens* from corneal ulcers grows on blood agar extensive liquefaction of Loeffler s serum slant typical diplobacillus

Other members of this group are not frequently encountered and have to be differentiated by special tests

D Large, capsule, free slimy growth fermentation of carbohydrates—*Klebsiella pneumoniae* Capsule swelling with specific antisera A B and C (Julianelle 1926) other strains have to be identified by biochemical tests

E Polar staining grows on ordinary media but may need blood, and one member requires special media (*Past tularensis*) ferments carbohydrates no coagulation of milk—Pasteurella

F Polar staining grows readily on ordinary media honeylike colonies on potato no fermentation of carbohydrates slow coagulation of milk—Malleomyces

G Characteristic spreading growth ferments carbohydrates splits urea motile with peritrichous flagella—Proteus

H No fermentation of carbohydrates motile with polar flagella many have soluble pigment—Pseudomonas

No pigment milk alkaline—*Alcaligenes faecalis*

I Pigmented colonies otherwise like colon group—Serratia

J Grows readily on simple alkaline media curved rods actively motile with polar flagella —Vibrio Identification of *Vibrio cholerae* by agglutination with O group I antiserum (Gardner and Venkatraman 1935 Linton 1940) and biochemical tests saccharose and mannite fermented arabinose not fermented no hemolysis of goat red blood cells—Pfeiffer s test with specific serum

4 Gram positive rods

A Nonmotile arranged in Chinese figures stain unevenly with bands and granules—*Corynebacterium*

These species of medical importance *C diphtheriae*—specific toxin *C pyogenes* and *C ulcerogenes* differentiated by biochemical reactions and animal inoculation

B Nonmotile frequently forming long filaments—catalase nega

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